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Is glycolysis a target to combat the malaria parasite? – Evaluation of the effect of 2,3-diphosphoglycerate on parasite susceptibility

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ABSTRACT

Malaria is a disease caused by an unicellular parasite of the genus *Plasmodium*, which is transmitted by the bite of female mosquito of the *Anopheles* genus. It occurs primarily in countries with tropical and subtropical climate and in 2017, around 219 million cases were reported and 435 000 people died due to the disease. The fight against malaria is a constant challenge, mainly due to the rapid emergence of resistance to new drugs, making the research of new antimalarials essential for its elimination.

Some host genetic factors are able to reduce the susceptibility to the disease. As a result of selective pressure exerted by malaria on the human genome, one in six people are affected by erythrocyte variants that confer some protection against the disease, such as sickle-cell anaemia, thalassemia and enzyme deficiencies such as glucose-6-phosphate-dehydrogenase (G6PD) and pyruvate kinase (PK), among others. Understanding the mechanisms underlying this protection may help finding new tools to fight this disease.

In PK deficiency case, the two major metabolic abnormalities are low ATP synthesis and increased 2,3-DPG concentration. This thesis aims to determine the effect of 2,3-DPG increase on parasite development in *in vitro* cultures of two *Plasmodium falciparum* strains – chloroquine-sensitive (3D7) and chloroquine-resistance (Dd2), through susceptibility assays (IC50), invasion and maturation assays and its effect on ATP production. Ultimately, on the long run, this may help to understand whether the increase of the intermediary human metabolite 2,3-DPG could be a potential antimalarial agent.

The results obtained show a decrease in parasitic growth with increasing concentration of 2,3-DPG, with IC50 values at 24, 48 and 72 hours ranging from 7.60 to 2.52 for 3D7 and 8.18 to 2.34 for Dd2. The invasion and maturation assays revealed a reduction in the invasion levels of erythrocytes in the presence of 2,3-DPG compared to the control (without the metabolite).

Keywords: Malaria, *Plasmodium falciparum*, pyruvate kinase deficiency, 2,3-diphosphoglycerate

RESUMO

A malária é uma doença causada por um parasita unicelular do género *Plasmodium,* transmitido pela picada de um mosquito fêmea do género *Anopheles.* Ocorre principalmente em países com clima tropical e subtropical. Em 2017, foram diagnosticados cerca de 219 milhões de casos e 435 000 pessoas morreram devido à doença. A luta contra a malária é um desafio constante, principalmente devido ao rápido surgimento de resistências a novos fármacos, sendo a pesquisa de novos antimalários essencial para a sua eliminação.

Existem factores genéticos do hospedeiro capazes de reduzir a susceptibilidade à doença. Como resultado da pressão selectiva exercida pela malária sobre o genoma humano, um em cada seis humanos no mundo é afectado por variantes nos eritrócitos que conferem alguma proteção contra a doença, como é o caso da drepanocitose ou anemia falciforme, talassemias e deficiências enzimáticas como glucose-6-fosfato-desidrogenase (G6PD) e piruvato cinase (PK), entre outros. A compreensão dos mecanismos subjacentes a esta protecção poderá ajudar a encontrar novas ferramentas para lutar contra esta doença.

No caso da deficiência em PK, as duas maiores anormalidades metabólicas são a baixa síntese de ATP e o aumento da concentração de 2,3-DPG. Esta tese tem como objetivo determinar o efeito do aumento de 2,3-DPG no desenvolvimento parasitário em culturas *in vitro* de dois clones de *Plasmodium falciparum* – sensível (3D7) e resistente (Dd2) à cloroquina, através de ensaios de suscetibilidade (IC50), invasão e maturação e do seu efeito na produção de ATP na glicólise de forma a perceber se o 2,3-DPG poderá ter potencial para ser transformado num agente antimalárico.

Os resultados obtidos evidenciam diminuição do crescimento parasitário com o aumento da concentração do 2,3-DPG, tendo os valores de IC50 calculados às 24h, 48h e 72h variado entre 7.60 e 2,52 para a estirpe 3D7 e 8,18 e 2,34 para Dd2. Nos ensaios de invasão–maturação observou-se uma redução dos níveis de invasão dos eritrócitos na presença de 2,3-DPG quando comparados com o controlo (sem o metabolito).

Palavras-chave: Malária, *Plasmodium falciparum,* deficiência em piruvato cinase, 2,3-difosfoglicerato.

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LIST OF ABREVIATIONS AND ACRONYMS

2,3-DPG – 2,3-diphosphoglycerate

ACTs – artemisinin-based combinations therapies

ADP – adenosine diphosphate

AFM – Atomic Force Microscopy

ATP – adenosine triphosphate

bp – base pairs

BPGM – 2,3- biphsphoglycerate mutase

CSP – circumsporozoite protein

CR1 – complement receptor 1

cRPMI – complete (with Albumax, HEPES, hypoxanthine and sodium bicarbonate) Roswell Park Memorial Institute 1640 Medium

CQ – chloroquine

DARC – Duffy antigen receptor of chemokines

EDTA – ethylenediamine tetraacetic acid

G6PD – glucose-6-phosphate dehydrogenase

GMS – Greater Mekong sub region

GSH – reduced glutathione

GSSG – oxidized glutathione

HbA – haemoglobin A

HbAS – haemoglobin AS (heterozygous for HbA)

HbC – haemoglobin C

HbE – haemoglobin E

HbSS – haemoglobin SS (homozygous for HbS)

HE – hereditary elliptocytosis

HEPES – 4-(2-hydroxyethyl)-1-piperazinnthanesulfonic acid

HS – hereditary spherocytosis

HSPGs – heparin sulphate proteoglycans

IC50 – half-maximal inhibitory concentration

NaCl – sodium chloride

$NADP^+$ – nicotinamide adenine dinucleotide phosphatase

NADPH – nicotinamide adenine dinucleotide phosphatase reduced form

NO – Nitric oxide

PBS – phosphate buffered saline

PCR – Polymerase chain reaction

PGK – phosphoglycerate kinase

PK – pyruvate kinase

PKD – pyruvate kinase deficiency

RBC – red blood cell

RDT – Rapid diagnostic test

RFU – Relative fluorescence units

RI – resistance index

SP – sulfadoxine-pyrimethamine

TRAP – thrombospondin-related adhesive protein

I – INTRODUCTION

1. MALARIA

Malaria is a disease caused by unicellular parasitic protozoans of the genus *Plasmodium*, transmitted by the bite of an infected female mosquito of *Anopheles* species. From the *Plasmodium* genus, six species can cause illness in humans namely, *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium knowlesi, Plasmodium ovale curtisi and Plasmodium ovale wallikeri.* (Sutherland *et al*., 2010; WHO 2015)

1.1. GEOGRAPHICAL DISTRIBUTION

The geographical distribution of the six *Plasmodium* spp. that infect humans varies. *Plasmodium knowlesi* is the most recent species that cause illness to humans, it is originally a monkey malaria parasite, and is circumscribed to the forested regions of South-East Asia, particularly the island of Borneo. (WHO, 2015) *Plasmodium malariae* cause the mildest infection and can be found worldwide but is more prevalent in West Africa. *Plasmodium ovale* is found in Asia and Africa, but it is also more prevalent in West Africa. (Phillips *et al*., 2017; Tuteja, 2007) The most widespread species is *P. vivax*, present in tropical and temperate areas, primarily Southeast Asia, Central and South America. *Plasmodium vivax* and *P. ovale* have a dormant liver stage – hypnozoite – that can remain in the liver for weeks or even years without being released to the bloodstream to infect red blood cells – erythrocytic schizogony phase (symptomatic). This, in the case of *P. vivax,* enables its presence on temperate regions allowing the species to survive cold seasons when the mosquito is not present and transmission is not possible. (Howes *et al*., 2016) As *P. vivax* depends on the Duffy antigen to invade red blood cells (RBC), in Africa where some regions have a high prevalence of Duffy negative phenotype, *P. vivax* is not endemic. *Plasmodium falciparum* is distributed worldwide and is the most pathogenic and deadly malaria species, as it causes severe malaria and has the highest prevalence in sub-Saharan Africa. (Phillips *et al*., 2017; Tuteja, 2007)

In 2017, *P. falciparum* was the most prevalent malaria species in the WHO African Region with 99.7% of estimated malaria cases, in the WHO regions of South-East Asia 62.8%, in the Eastern Mediterranean 69% and the Western Pacific 71.9%, with the only exception being in the WHO Region of the Americas where *P. vivax* represent

74.1% of malaria cases. Two hundred and nineteen million was the estimated total of malaria cases in 2017, 20 million fewer than in 2010. However, there was no significant further reduction on the number of cases between 2015 and 2017. The same pattern occurred with the number of deaths from malaria, 435 000 deaths in 2017, reducing when compared with 2010 (607 000 deaths), but slowing its rate since 2015. (WHO, 2018)

The most vulnerable group are children under age of five, who accounted for 61% of deaths worldwide, the majority of cases (80%) occurs in sub-Saharan Africa and India. The global distribution of malaria indigenous cases in 2000 and their status in 2017 is shown in Figure 1. (WHO, 2018)

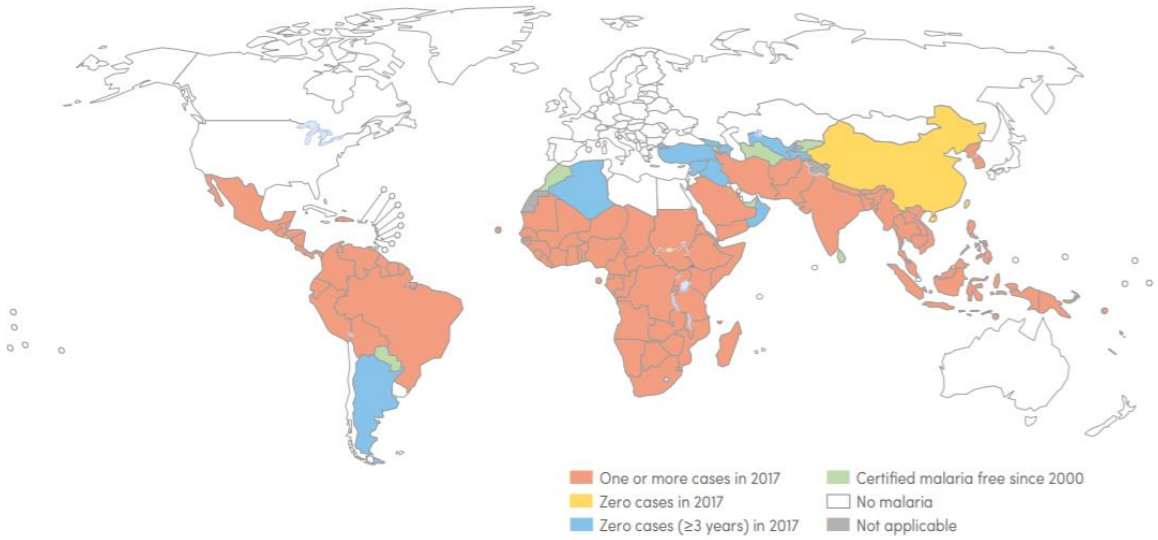

Figure 1 – Countries with indigenous malaria cases in 2000 and their status in 2017. (WHO, 2018)

1.2. PREVENTION

Malaria is a treatable and preventable disease; the goal is to reduce transmission, both by applying control measures against the vector as well as by chemoprevention (reducing the infection reservoir and providing drugs that suppress infection to specific high-risk groups). (WHO, 2015)

To prevent transmission of malaria, vector control measures such as long-lasting insecticidal nets (LLINs) and indoor residual sprayings (IRS) are applied, which are

successful when coverage is enough. Other supplementary interventions may be applied, such as regular application of biological or chemical insecticides to water bodies (larvicide), or the use of personal protection (insecticide-treated clothing or topical repellents), being the last measure more targeted to travellers. (WHO, 2019a) However, to achieve the goal of eliminating the disease, vector control must be combined with the use of antimalarial medicines for prophylaxis and for preventive treatment. Chemoprevention with sulfadoxine-pyrimethamine (SP) is strongly recommended as an Intermittent Preventive Treatment (IPT) of malaria in pregnancy, in infants and in seasonal malaria, combined with amodiaquine in the sub-Sahel region of Africa. (WHO, 2015)

Another priority to decrease clinical cases and mortality caused by malaria is the development of a malaria vaccine. Currently several vaccines with different targets are in different stages of study, some focus on the pre-erythrocyte stage, others on the blood stage or in transmission blocking. (Cockburn & Seder, 2018) In late April 2019, RTS,S/AS01 vaccine started the phase 4 studies, where young children from Ghana, Malawi and Kenya are receiving the vaccine (composed of 4 doses). It is estimated that each year at least 360 000 children will be immunized. (WHO, 2019b) RTS,S/AS01 is a recombinant protein that targets the circumsporozoite protein of *P. falciparum*, expressed by the malaria parasite at the pre-erythrocyte stage, in which part of the circumsporozoite sequence is coexpressed with fused and free hepatite B surface antigen 2,3 and formulated with the AS01 adjuvant. Phase 3 studies reported that this vaccine confers a 30% protection against clinical infection over 4 years. (Rts, 2015)

1.3. SYMPTOMS AND DIAGNOSIS

Malaria is normally classified as uncomplicated or severe. The symptoms occur when *Plasmodium* spp. invades erythrocytes and the pyrogenic threshold is attained, and they are shaking chills, muscle aches and low fever, if not diagnosed it can evolve to sweats, high fever and exhaustion. Determination of pyrogenic threshold (in periods of low and high transmission) is a good tool to assess changes in the epidemiology of the disease. (Dollat *et al*., 2019; Phillips *et al*., 2017) In the case of *P. falciparum* the disease can progress for severe malaria and can be fatal. The most common symptoms of severe

malaria are severe anaemia and capillaries obstruction due to the adhesion of parasitized erythrocytes to endothelium, which leads to organ damage as in cerebral malaria. (Phillips *et al*., 2017; WHO, 2015)

To confirm diagnosis, patients with suspected malaria should have their blood tested by immunochromatographic rapid diagnostic test (RDT) or by light microscopy examination of thick and thin blood smear. (WHO, 2015)

1.4. TREATMENT

A major problem when trying to combat malaria is when strains of the parasite start to develop resistance to antimalarials. Over time several drugs have been developed to combat infection by malaria parasite. The first one and one of the most effective to date was quinine; it was first isolated in 1820 and the first cases of resistance were reported in the 1980s. Despite of not being used as a front-line treatment since 2006 it is still used to treat cases of severe malaria in areas where artemisinin is not available. (Tse *et al*., 2019)

Chloroquine is an antimalarial used in the 1940s but a decade later the first cases of resistance were reported. Over the years, several strains of malaria have developed resistance to chloroquine and nowadays strains such as W2 or Dd2 are used to evaluate the potential of new drugs as antimalarials. (Tse *et al*., 2019) Chloroquine is still the first-line treatment against *P. vivax* infections; however, resistance is increasing. This antimalarial acts in the digestive vacuole – when inside it binds to a heme dimer (hematin) preventing the detoxification of free heme, resulting in the accumulation of heme monomers that permeabilize the membrane leading to the dead of the parasite. Structurally related quinolone drugs, such as quinine, chloroquine, mefloquine and mepacrine, act in the disruption of haemoglobin digestion. (Petersen *et al*., 2011)

Mefloquine and piperaquine were developed in response to the emergence of chloroquine resistant strains, and as they have similar structure to chloroquine, it was postulated that they would have a similar mechanism of action. The use of piperaquine as a monotherapy in the 1970s also originated resistant *P. falciparum* strains. In the case of mefloquine, it is no longer commonly used alone due to its toxicity for the central nervous system reported in many of users. Nowadays, these antimalarials are used as partner drugs

in an artemisinin-based combination therapy due to their extended half-life (approximately 5 weeks for piperaquine and 3 weeks for mefloquine). (Petersen *et al*., 2011)

Currently, the artemisinin-based combinations therapies (ACTs) are the recommended treatment for malaria (Figure 2). These are the most effective antimalarial medicine available today due to the combination of a semi-synthetic derivate of artemisinin (short-term acting) with a partner drug of a different chemical class (long-term acting and different mechanisms of action). (Petersen *et al*., 2011) The most common derivatives are artesunate, artemether and arteether, which are prodrugs that are transformed in dihydroartemisinin. The most accepted theory concerning the mechanism of action is that the molecule is activated by haem generation free radicals, which will damage proteins necessary for parasite survival. (Tse *et al*., 2019)

Amodiaquine is also used as a partner drug and has a mechanism of action similar to that of chloroquine. This ACT is used to treat uncomplicated *P. falciparum* infections. Other partner drugs have a different mechanism of action. Lumefantrine and pyronaridine are suggested that inhibit nucleic acid and protein synthesis through the inhibition of β-haematin formation by complexation with haemin. (Tse *et al*., 2019)

A combination of pyrimethamine and sulfadoxine is also used to treat malaria. These drugs have a different mechanism of action from those mentioned above as both act in the folate biosynthesis pathway in the parasite – the first acts in the inhibition of dihydrofolate reductase, and the second inhibits dihydropteroate synthetase. (Tse *et al*., 2019) Another combination of drugs used to treat malaria is proguanil and atovaquone that act as synergists, due to the different mechanisms of action. Proguanil does not act on their target when combined with atovaquone but reduces the concentration of atovaquone needed for treatment. The last drug will thus block the mitochondrial electron transport once it acts as an inhibitor of cytochrome bc1 complex. (Tse *et al*., 2019)

For the treatment of *P. vivax* hypnozoite liver stages there are only two antimalarials primaquine and more recently tafenoquine. (Petersen *et al*., 2011; Tse *et al*., 2019)

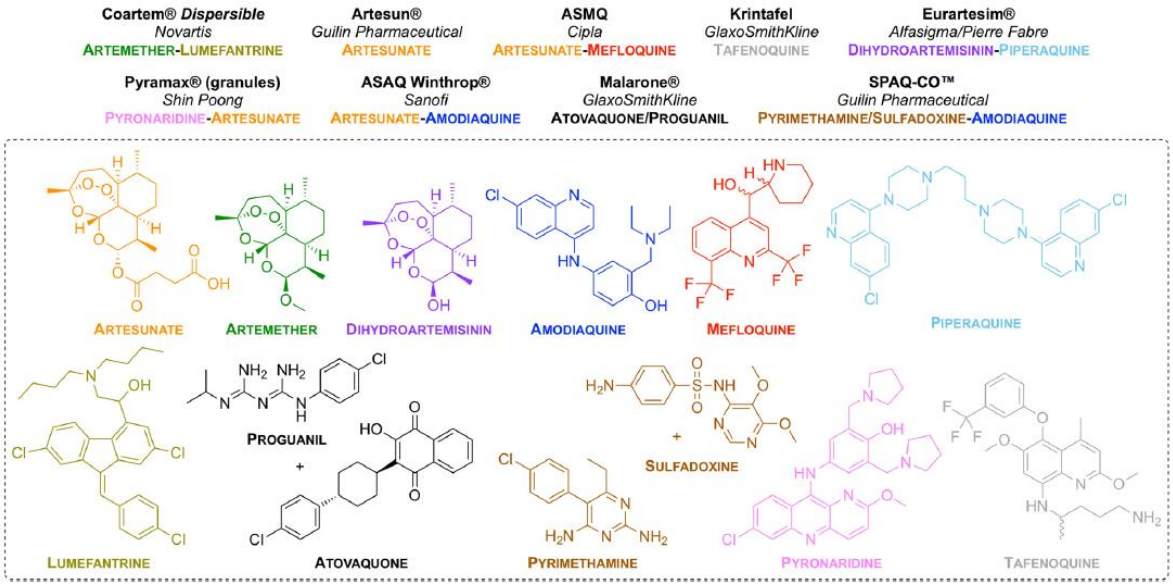

Figure 2 - Drug combinations recommended for treatment. Brand name of the drug (in bold), partnered company (in italics) and drug combination (colour-coded to the structures) are listed. (Tse *et al*., 2019)

ACTs increase treatment efficacy and reduces the emergence of drug resistant parasites, with an overall efficacy rates greater than 95% outside the Greater Mekong sub region (GMS), where treatment failure with several ACTs has already been reported. (Petersen *et al*., 2011) In many areas of Asia, the ACT dihydroartemisinin and piperaquine is the first line of treatment. However, a strain – KEL1/PLA1 became resistant to this treatment and a study from 2018 alerts for a worse scenario in the last few years. This strain that in 2009 was only found in western Cambodia, has now a prevalence higher that 50% in GMS (except for Laos), and with a prevalence higher than 80% in northeast Vietnam and Thailand. These results alert to the importance of surveillance in order to eliminate the spread of multiresistant drug *P. falciparum* strains. (Hamilton *et al*., 2019)

The emergence of resistance depends on the mutation rate of the parasite, the fitness cost associated with resistance mutations, parasite load, the strength of drug selection and the treatment compliance. Good practices of treatment are essential to prevent and/or delay the emergence of new drug resistances as well as monitoring drug resistance in clinical settings.

1.5. *PLASMODIUM* LIFE CYCLE

During a blood meal of an infected female mosquito of the *Anopheles* genus, sporozoites are injected into the mammalian host. The sporozoites then travel through the skin into the bloodstream until the liver where they infect hepatocytes. However, not all sporozoites enter in the blood circulation. Some enter the lymphatic circulation until they reach the lymph node where they are degraded, others remain at the injection site in the skin where they will be eliminated by phagocytes, and this contributes to the protective immune responses against *Plasmodium* spp.. (Silvie *et al*., 2008) The invasion of the hepatocytes is mediated by a co-receptor on sporozoites involving the thrombospondin domains on thrombospondin-related adhesive protein (TRAP) and on the circumsporozoite protein (CSP) that binds to the heparin sulphate proteoglycans (HSPGs) on hepatocytes in the region in apposition to sinusoidal endothelium and Kuppfer cells. In the liver cells, sporozoites replicate and divide mitotically, when the infected hepatocyte ruptures, merosomes – parasite-filled vesicles, are released into the blood circulation. Presumably, merosomes rupture in the microvasculature of the lungs, releasing tens of thousands of merozoites that invade erythrocytes, beginning the asexual reproductive stage. (Miller *et al*., 2002; Prudêncio *et al*., 2011)

To invade erythrocytes, merozoites formed a tight junction between the erythrocyte membrane and the parasite. The junction moves from the apical to the posterior end of the merozoite in a series of events powered by the parasite action-myosin motor. As the merozoite enters the host cell, a parasitophorous vacuole is created to separate the parasite from the host cytoplasm. (Cowman & Crabb, 2006) Upon entry into the erythrocyte, ring stage parasite begins to modify their host cell through export of remodelling factors and a series of variable antigens. Parasites ingest the haemoglobin present on the erythrocyte and to avoid its toxic effect, trophozoite stage parasite polymerize the haem into hemozoine, using the remaining components for nutrients. After the polymerization of haem, trophozoite go through a series of nuclear divisions to form multinucleated schizonts. Schizonts undergo cell division to form multiple merozoites, the erythrocyte rupture and the merozoites reinvade new RCB. (de Koning-Ward *et al*., 2016; Stanway *et al*., 2009; van Dooren *et al*., 2005)

After each cycle, a subpopulation of blood stage parasites differentiates to the sexual form known as gametocytes. Host or environmental factors can trigger gametocytogenesis, the mechanism by which the malaria parasite switches from asexual blood stages to gametocytes. (de Koning-Ward *et al*., 2016) When a female *Anopheles* spp. mosquito has its blood meal from an infected mammalian, it ingests female and male gametocytes (macrogametocytes and microgametocytes, respectively), leading to the development of mature gametes and subsequently fusion of the gametes to produce a zygote. The zygote undergoes meiosis and elongation to produce a motile ookinete, which penetrates the midgut wall and develop into an oocyst. After maturation, the oocyst migrates to the salivary glands and when the female mosquito takes another blood meal, the life cycle repeats (Figure 3). (Guttery *et al*., 2015; Sinden, 2015)

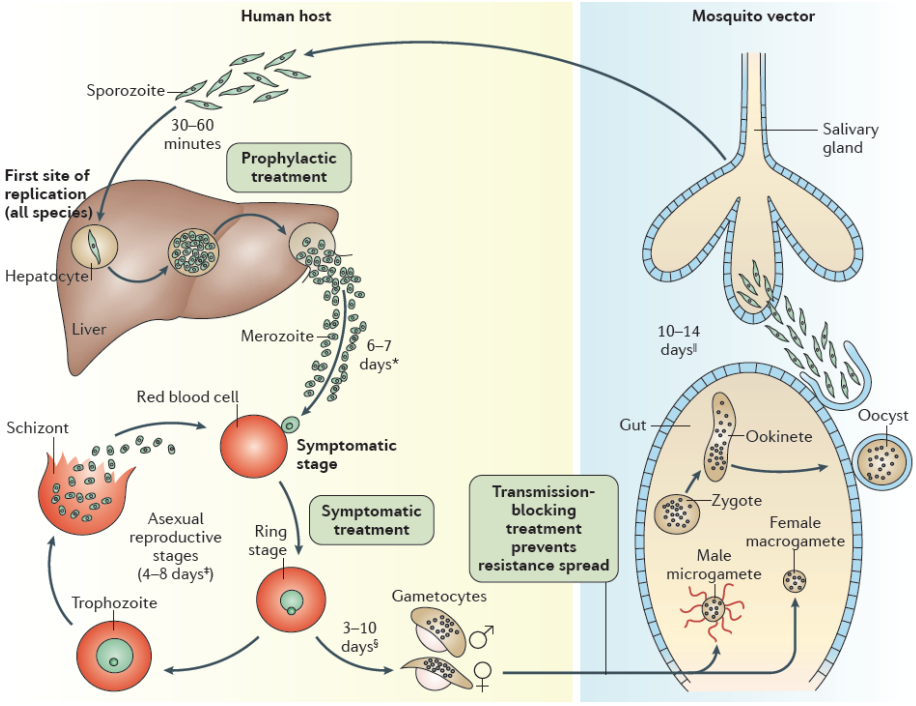

Figure 3 – *Plasmodium* spp. life cycle. During a blood meal, the vector transmits the *Plasmodium* spp. parasite in the sporozoite stage to the host. Sporozoites then invade hepatocytes, where they replicate and divide as merozoites. When hepatocytes ruptures, the merozoites invade erythrocytes in the blood stream and begin the asexual reproduction (symptomatic stage). Inside the erythrocyte the merozoite transforms into a ring stage, which will grow to trophozoite stage that will mature to schizont. Schizonts undergo cell division to form multiple merozoites, the erythrocyte rupture and the merozoites reinvade erythrocytes. Some blood stage parasites differentiate to the sexual form (gametocytes). When a female mosquito has a blood meal from an infected mammalian, ingests gametocytes, leading to the development of mature gametes and subsequently fusion of the gametes to produce a zygote. The zygote undergoes meiosis and elongation to produce a motile ookinete, which penetrates the midgut wall and develop into an oocyst. After maturation, the oocyst migrates to the salivary glands and when the female mosquito takes another blood meal, the life cycle repeats. (Phillips *et al*., 2017)

2. THE HOST CELL - ERYTHROCYTE

The erythrocyte is the 'centre stage' for the asexual development of the malaria parasite. Within the RBC cells, repeated cycles of parasitic development occur with precise periodicity, and at the end of each cycle, hundreds of fresh daughter parasites are released that invade more RBC.

The erythrocytic cycle described in the previous section, occurs every 24 hours in case of *P. knowlesi*, 48 h in case of *P. falciparum*, *P. vivax* and *P. ovale* and 72 h in case of *P. malariae*. During each cycle, each merozoite grows and divides within the vacuole into 8–32 (average 10) fresh merozoites, through the stages of ring, trophozoite, and schizont. At the end of the cycle, the infected red cells rupture, releasing the new merozoites that in turn infect more RBCs and parasitaemia increases. (Greenwood *et al.* 2008) Due to the lack of nuclei and most organelles (for example mitochondria, reticulum), erythrocytes cannot reproduce themselves or maintain their structure for very long, having an average life span of approximately 120 days. Erythropoiesis (erythrocyte production) is regulated by an hormone – erythropoietin – secreted into the blood by a group of hormone-secreting connective tissue cells in the kidney. This hormone stimulates the bone marrow to produce reticulocytes (young erythrocytes with ribosomes) and their differentiation into mature erythrocytes. The destruction of damaged erythrocytes occurs in the liver and spleen. (Widmaier *et al*., 2014)

The absence of a nucleus gives erythrocytes its biconcave shape, which provides an optimal area for respiratory changes. The RBC has a surface capable of deforming itself to pass through microcapillaries; this is achieved by their membrane composition. (van Wijk & van Solinge, 2005). The erythrocyte membrane consists of a plasma membrane envelope composed of amphiphilic lipid molecules anchored to a two-dimensional network of skeletal proteins through transmembrane proteins in the lipid bilayer (Figure 4) (Mohandas & Gallagher, 2008).

The lipid bilayer is composed of cholesterol and phospholipids; while the first is evenly distributed, the second is asymmetrically disposed. In the outer monolayer, phosphatildylcholine and sphingomyelin are predominant, while most phosphatildylethanolamine and phosphatildylserine are present in the inner monolayer. To maintain the phospholipid asymmetry energy-independent and energy-dependent

phospholipid transport proteins are present in the membrane, for example “flippases” transport phospholipids from the outer to the inner monolayer, while “floppases” transport phospholipids from the inner to the outer monolayer against the concentration gradient using energy. The membrane proteins have different functions as they can act as a transporter, signal receptors or adhesion proteins. The membrane transporters include ion, water, urea, or glucose transporters. (Mohandas & Gallagher, 2008)

Erythrocyte membrane integrity and deformability are mainly maintained by the skeleton membrane and its structural flexibility is provided by the helical linkers between the spectrin repeats. The spectrin heterodimers are connected to a junction complex containing protein 4.1R, actin oligomers, adducin, tropomodulin, dematin, p55 and tropomodulin. Ankyrin has a spectrin-binding domain and an amino-terminal domain that interacts with band 3, a transmembrane protein, which promotes tetramerization of this protein and decreases diffusional mobility, this interaction is stabilized by protein 4.2 and Rhesus proteins. A ternary complex formed by protein 4.1R, p55 and the cytoplasmic domain of glycophorin C provides a second point of attachment to the plasma membrane (Figure 4) (Maier *et al*., 2009). These two protein complexes provide important points of linkage between the lipid bilayer and the spectrin-based skeleton network, which allows the RBCs to maintain its membrane mechanical stability. The primary constituents of membrane stability are protein 4.1R, actin and spectrin. The interaction between dimer-dimer it is dynamic, it opens reversibly under tensile forces imposed by deformation. (Mohandas & Gallagher, 2008)

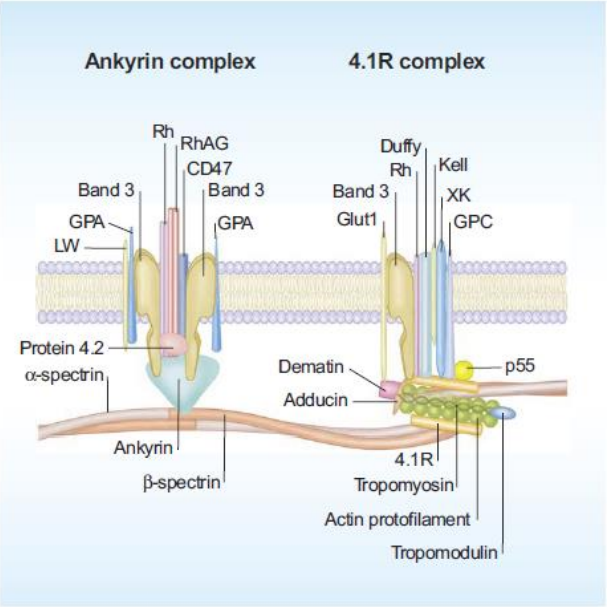

Figure 4 - A schematic representation of red blood cell membrane. The erythrocyte membrane consists of a plasma membrane envelope composed of amphiphilic lipid molecules anchored to a 2-dimensional network of skeletal proteins through transmembrane proteins in the lipid bilayer. (Mohandas & Gallagher, 2008)

Inherited erythrocyte disorders can alter cell membrane and cell function, normally these polymorphisms result in a decreased of cell deformability. Mutations in ankyrin, band 3 and β-spectrin genes are responsible for hereditary spherocytosis (HS). This disorder can reduce the spectrin concentration to 80% (mild HS) or to 50% (severe HS) when compared to a healthy RBC resulting on a smaller shape diameter of the RBC and decreased deformability. Mutations in α-spectrin, β-spectrin, glycophorin C and protein 4.1 genes are responsible for hereditary elliptocytosis and result in a decreased deformability of the RBC. (Suresh, 2006)

In the next section, we will present some polymorphisms that confer resistance against malaria, most of them related to the main host cell, the erythrocyte.

3. POLYMORPHISMS THAT CONFER RESISTANCE AGAINST MALARIA

3.1. HAEMOGLOBINOPATHIES

Genetic disorders of haemoglobin (Hb) include α- and β-thalassaemias, HbS, HbC and HbE and they are the most prevalent monogenic disorders in humans. Haemoglobinopathies have a higher frequency in regions where malaria is endemic; this fact has been credited to a selective pressure exerted by the malaria parasite on the human host genome through balancing selection. (Min-Oo & Gros, 2005)

3.1.1. Thalassaemia

Thalassaemia are widely distributed around the world and are the result of an alteration in the synthesis of the α- or β-globin chains.

In the case of α-thalassaemia, it is the result of a deletion of the duplicated α-globin genes in chromosome 16 that leads to a decreased synthesis of a α-globin chain. For homozygotes it is lethal, but for heterozygotes is responsible for a mild haemolytic state and is associated with a reduced erythrocyte survival which leads to a higher proportion of young erythrocytes in circulation.

β-thalassaemia results from a decreased β-globin and an excess synthesis of α-globin. Homozygotes are associated with severe anaemia and it leads to dead within the first two years of life, in the case of heterozygous state, it is associated with morphological changes in erythrocytes and with mild anaemia. It is described that infected thalassaemic RBCs are more susceptible to phagocytosis by monocytes and show decreased rosette formation, associated with severe forms of malaria. (Min-Oo & Gros, 2005) In β-thalassaemias early ring-stage infected RBCs have an increased phagocytosis caused by an increased binding to complement 3 and IgG. (Ayi *et al*., 2004)

3.1.2. HbS (sickle cell disease)

HbS is a result of a mutation in β-globin chain and have a high prevalence in tropical Africa. Heterozygous individuals (HbAS), are healthy and the presence of sickle-

cell trait (allele HbS) gives them protection against severe forms of malaria. It is showed that infected ring-stage RBCs have a higher rate of phagocytosis. (Ayi *et al*., 2004) In both homozygous (HbSS) and heterozygous RBCs, there is a reduced invasion and maturation of the parasite. (Ayi *et al*., 2004)

3.1.3. HbC and HbE

Haemoglobin C (HbC) is almost exclusive of Western Africa and is a result of a point mutation. Several mechanisms have been proposed for HbC, and it is known that both heterozygous and homozygous individuals have a reduced risk of having non-severe and severe infections. Haemoglobin E (HbE), more common in Southeast Asian, is also a result of a point mutation. Homozygous RBCs present a lower volume and lower haemoglobin concentration leading to anaemia. This haemoglobinopathy confers protection against severe malaria. (López *et al*., 2010)

3.2. IMMUNOGENETIC VARIANTS

Some mechanisms that confer protection against malaria involve immunogenetic variants such as chromosome 5q31–q33, HLA-Bw53/HLA-DRB1*1302-DQB1*0501, complement receptor 1 (CR1) polymorphism that is associated with reduced susceptibility against severe malaria or polymorphism in the promoter region of nitric oxide synthase 2 that does increase NO production and may also be a resistance mechanism against malaria. (López *et al*., 2010)

3.3. ERYTHROCYTE STRUCTURAL POLYMORPHISMS

3.3.1. Duffy antigen

Duffy antigen receptor of chemokines (DARC) is expressed on the surface of RBCs and other cell types. A lack of Duffy antigen in erythrocytes due to a point mutation, leads to resistance against *P. vivax* infection as this receptor is essential for the ligation of *P. vivax,* mediated by the parasite Duffy binding protein. Duffy negativity is more common in Papua New Guinea and West Africa. (López *et al*., 2010) However,

cases of infections in Duffy antigen negative individuals have been reported, suggesting an alternative mechanism of invasion in some strains. (Mendes *et al.*, 2011)

3.3.2. Ovalocytosis (band 3 variants)

Ovalocytosis is asymptomatic and endemic in Southeast Asia, and seems to be protective against cerebral malaria. This erythrocyte polymorphism is an uncommon variant of hereditary elliptocytosis (HE) and is caused by a deletion in the gene that encodes the RBC membrane protein band 3. (López *et al*., 2010)

3.4. ENZYMOPATHIES

3.4.1. G6PD deficiency

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that catalyses the first step in the hexose monophosphate pathway leading to the synthesis of pentose phosphate. It also catalyses the conversion of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to its reduced form (NADPH), which protects RBCs from oxidative damage. (López *et al*., 2010) Deficiency in the activity of this enzyme is a disorder extremely frequent in tropical countries and in the Middle East. Mutations in the gene, which comprises 13 exons, vary greatly between populations. (Min-Oo & Gros, 2005)

G6PD deficient RBCs infected with early ring-stage parasite are more susceptible to phagocytosis by monocytes, simultaneously increased binding of IgG and complement C3 to infected RBCs (Ayi *et al.*, 2004). Due to the low levels of reduced glutathione (GSH) and consequently an increased GSSG/GSH ratio an indicative of oxidative stress, G6PD deficient cells are more prone to changes in their cell membrane. At the mature stage parasite, the production of haemozoin (crystal product of the haem degradation) inhibits function of the monocyte such as oxidative bursts and subsequent phagocytosis. (Min-Oo & Gros, 2005)

3.4.2. PK deficiency

Pyruvate kinase (PK) deficiency is a hereditary form of non-spherocytic haemolytic anaemia, characterized by chronic haemolysis and anaemia. Three of the most prevalent mutations in patients with PK deficiency are 1529A (most common in northern and central Europe), 1456T (most common in southern Europe), and 1468T (most common in Asia). (Beutler & Gelbart, 2000)

PK deficiency is the most frequent abnormality in glycolysis and is inherited as an autosomal recessive trait, caused by loss-of-function mutation in *pklr*. (Durand & Coetzer, 2008)

The *pklr* gene codes for the erythrocyte PK, which catalysis the conversion of phosphoenolpyruvate to pyruvate, producing a molecule of ATP by transferring the phosphate group of phosphoenolpyruvate to an ADP molecule. PK is a regulatory enzyme active as a tetramer and four different isozymes are express in mammals. (Durand & Coetzer, 2008) The two isozymes PK–R, only express in RBC and PK–L, predominantly expressed in the liver, are transcribed from a single gene (*pklr*), located on chromosome 1q21, by the use of alternative promoters. The other two types of isozymes PK–M1 and PK–M2 are transcribed from a single gene PKM by alternative splicing. While PK–M1 is expressed in heart, brain and skeletal muscle, PK–M2 is expressed in adult tissue, such as leucocytes and platelets, and in early foetal tissue. (van Wijk & van Solinge, 2005)

PK deficiency has a protective effect against the malaria parasite in RBCs. The mechanism of protection may include a reduced invasion of erythrocytes, in case subjects with homozygous mutation and a higher macrophage clearance on ring stage infected RBCs for both homozygous and heterozygous. These mechanisms may cause an overall reduction of RBCs infected. (Ayi *et al*., 2008)

The parasite depends strongly on glycolysis and a loss of function of *pklr* impairs this metabolic pathway. Infection by *Plasmodium* spp. has been shown to increase glucose consumption of RBCs, resulting in a substantial increase in glycolytic enzyme levels activity of the parasite. (Oelshlegel *et al*., 1975) A decrease in ATP may lead to chances in the integrity of the RBC membrane, which may explain the early destruction by the reticulo-endothelial system. (Min-Oo & Gros, 2005) PK deficiency cause two major abnormalities that may be involved in the mechanism of protection against malaria

infection and that are presented in more detail in the next section: the increase of 2,3-DPG and the decrease of ATP production. (van Wijk & van Solinge, 2005)

4. 2,3 – DIPHOSPHOGLYCERATE (2,3-DPG)

Due to the lack of mitochondria and therefore the Krebs cycle, the only form that RBCs have to produce energy is through glycolysis. In mammalian RCBs, glycolysis has a bypass, the Rapoport-Luebering shunt that is responsible for the synthesis of the metabolite 2,3-DPG (Figure 5). This metabolite function is to regulate the affinity between oxygen and haemoglobin. (van Wijk & van Solinge, 2005) The normal values of 2,3-DPG in RBCs are between 3.6 and 5 mM. (Asakuta *et al*., 1966)

Enzyme deficiencies near the 2,3-DPG step (ie PK deficiency) lead to increased 2,3-DPG levels, as a result of bidirectional reaction steps that originate accumulation of upstream glycolysis products. The increased 2,3-DPG levels result in a decreased oxygen affinity of haemoglobin so that oxygen is more readily transferred to tissues. Additional consequence of the accumulation of 2,3-DPG is the decreased in the ATP synthesis, due to the higher activity of the Rapoport-Luebering shunt bypassing the synthesis of ATP catalysed by phosphoglycerate kinase (PGK). (van Wijk & van Solinge, 2005) In high altitudes, the levels of 2,3-DPG in RBCs increase in order to increase oxygen releasing to the tissues. This also results in a decrease in ATP production. However, in normal conditions, 2,3-DPG levels are regulated by biphosphogycerate mutase (BPGM) and phosphatase. Increasing concentrations of glucose in the medium compensates the inhibition of 2,3-DPG production. (Mehta *et al*., 2005) *Plasmodium falciparum* contrary to mammalian RBCs thus not possess a BPGM for 2,3-DPG synthesis, so for *P. falciparum* this metabolite does not have a role for parasite survival. It was showed before that infected RBCs that coexists with uninfected RBCs in the same culture have a lower activity of BPGM, the enzyme responsible for the synthesis of 2,3-DPG. (Kruckeberg *et al.*, 1981)

2,3-DPG also affects the stability of RBCs membrane. The addition of 2,3-DPG to the medium affects spectrin dissociation with a maximum of 60% dissociation with a concentration of 2,3-DPG of 4 mM, in contrast with 20% dissociation of spectrin in RBCs

in a medium without addiction of 2,3-DPG. The alteration of pH of the medium is a major factor that contributes to the transition of spectrin tetramer to dimer, more specifically pH above 8 increases dissociation of spectrin from the shell. The maintenance of 2,3-DPG levels is essential for the RBC membrane maintain its deformability. (Sheetz & Casaly, 1981)

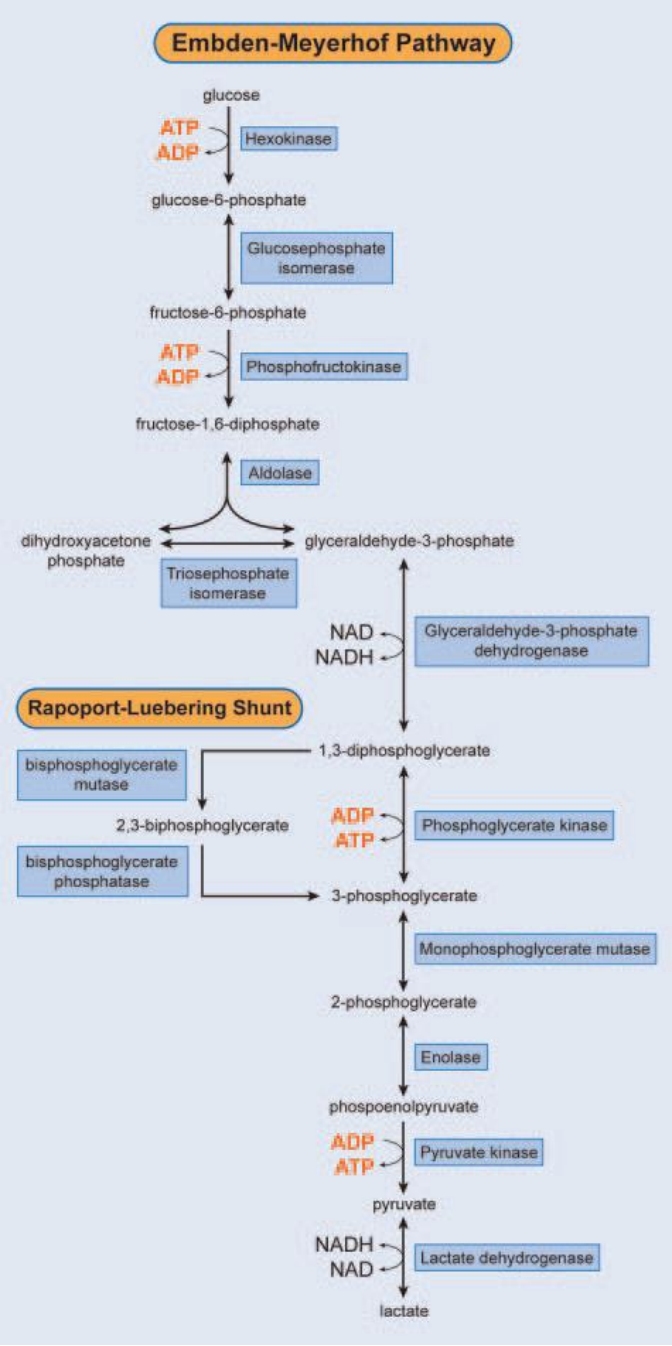

Figure 5 - Schematic representation of glycolytic pathway in mammalians, the Embden-Meyerhof patway and the Rapoport-Luebering shunt. (van Wijk & van Solinge, 2005)

II – JUSTIFICATION AND OBJECTIVES

The fight against malaria continues to be challenging, the rapid emergence of resistant strains to new antimalarials and the lack of an high efficiency vaccine makes the development of new tools to battle the disease an imperious task. The dependence of the parasite on RBCs – host cell – may provide targets for new approaches. Due to selective pressure that malaria has exerted through time on the human genome, human variants that protect against the disease emerged. New approaches targeting the host – host directed therapies – that mimic this natural protective effect can be promising.

A likely target on the host cell is glycolysis. The parasite possesses its own glycolytic enzymes with the exception of BPGM, the enzyme responsible for the synthesis of 2,3-DPG. The absence of this enzyme and consequently the absence of 2,3-DPG in the parasite raises the question: may it be harmful for the parasite? In addition, an increase in the concentration of this metabolite is one of the abnormalities caused by pyruvate kinase deficiency, so to explore if this effect could contribute to the resistance provided by PK deficiency against malaria infection may be interesting.

Therefore, the main goal of this thesis is to evaluate the effect of 2,3-DPG on the malaria parasite. This was investigated by performing parasite susceptibility assays in two strains of *P. falciparum*, chloroquine sensitive (3D7) and chloroquine resistant (Dd2), in order to calculate IC50 values and the ratio of invasion and maturation. The effect of 2,3-DPG on the synthesis of ATP was also assessed by calculating the ATP levels in RBCs in both assays.

This thesis is performed within the project “When the host cell is not so cosy anymore… A drop off in energy or an increase in toxicity?” that has as ultimate goal to evaluate if 2,3-DPG could be used as an antimalarial agent in the future.

III – MATERIALS AND METHODS

1. EXPERIMENTAL DESIGN

The effect of the metabolite 2,3-DPG on the parasite development was assessed using *in vitro* cultures of *P. falciparum*. These and all subsequent assays imply the use of RBCs provided by voluntary donors (section 2.1). To exclude any blood variants that could influence and bias the outcome of the parasite susceptibility assays, molecular diagnosis of the most common polymorphisms in Portugal was performed (HbAS, *pklr* and *g6pd* genes) (section 3). The respective genes were amplified by polymerase chain reaction (PCR) for the polymorphisms and by multiplex PCR for HbAS. In the case of HbAS, the gene amplification results in a different band pattern regarding the presence or absence of the mutation. However, for the polymorphisms in *pklr* and *g6pd* genes, further verification was needed. For *g6pd* gene, restriction analysis was performed and for *pklr*, the amplified fragment was sequenced. After verification of the absence of any blood variant, we proceeded to the parasite susceptibility assays.

To evaluate the effect of 2,3-DPG on the parasite (section 4), parasite susceptibility assays were performed with two strains of *P. falciparum*: 3D7 and Dd2, chloroquine sensitive and resistant strains, respectively. SYBR Green assays using unsynchronized cultures were performed to determine the IC50 value at 24, 48 and 72h. However, due to the lack of consistent results the IC50 determination and the following assays were performed using synchronized cultures. After the IC50 determination, three concentrations of 2,3-DPG were chosen to proceed to invasion-maturation assays, where cultures were monitored by thin blood smears every 24 hours until lysis of the culture. For better understanding of the effect of 2,3-DPG, ATP assays were carried out simultaneously as SYBR Green assays (IC50 determination) and invasion-maturation assays.

2. BIOLOGICAL MATERIAL

2.1. BLOOD DONORS

Healthy RBCs were obtained from haematological healthy individuals, voluntary staff donors. Venous blood collected by venepuncture in evacuated tubes containing 50 μL of 1 g/dL EDTA (ethylenediamine tetraacetic acid). Blood sample was washed by

centrifugation (Centrifuge 5810 R, Eppendorf, Hamburg, Germany) for 5 minutes at 670.8 x g. The supernatant was removed and approximately 5 ml of PBS (Sigma-Aldrich, St. Louis, MO, USA) were added, the blood sample was washed by centrifugation (Centrifuge 5810 R, Eppendorf) for 5 minutes at 670.8 x g, this procedure was repeated four more times. To obtain a haematocrit of 50%, the same volume of cRPMI was added to the pellet of RBC and the RBCs were stored at 4° C. Ideally, these cells will be used within 24h maximum after withdrawal after a broad written informed consent be obtained and discarded after two weeks maximum.

The protocol is included in the on-going project PTDC_BIA-CEL_28456_2017, which was approved by the Ethical Committee of IHMT; all procedures follow the European Directives and national rules as established in the community directives 2004/23/CE, 2006/17/CE, 2006/86/CE and the General Data Protection Regulation (GDPR), 2016/679, 27 April. Each donor was informed about the nature and main objectives of the study and was told that participation was voluntary. Blood samples were collected after written informed consent (in Annex 1). A code number was assigned to each participant and confidentiality is maintained regarding all information obtained. In order to exclude any blood variant, an aliquot of 1 ml of full blood was kept at time of collection for consequent DNA preparation and molecular diagnosis of HbAS and polymorphisms of *g6pd* and *pklr* genes.

2.2. *PLASMODIUM FALCIPARUM* STRAINS

In vitro cultures of *P. falciparum* strains 3D7 and Dd2 (sensitive and resistant to chloroquine and mefloquine, respectively), were used. Both were stored in liquid nitrogen and thawed for this study.

3. BLOOD GENOTYPING

For the molecular diagnosis of HbAS and polymorphisms of *g6pd* and *pklr* genes, PCR based techniques were used. First, DNA from blood samples from the different donors was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The DNA concentration was calculated by

measuring the absorption at 260 nm using Nanodrop Spectrophotometer ND 1000 (Thermo Fisher Scientific, Waltham, MA, USA).

3.1. HbB

For the detection of sickle cell trait - allele HbS, resulting from a mutation A>T c.6 in the β-globin gene (HbB), a bi-ASA-PCR (bidirectional single tube allele-specific amplification) was performed using an adaptation of the technique described by Waterfall and Cobb (2001).

PCR reaction was performed in a final volume of 25 µl with 2.5 units/µl of HotStarTaq® (Qiagen), 1 µM of the primers WT-AS; WT-CP517; MUT-AS; MUT-CP267 listed in Table 1, 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 1x HotStartTaq Master Mix and 10 ng/µl DNA from the blood samples. The program used in the thermocycler (T1 thermocycler Biometra, Göttingen, Germany) was as follows: initial denaturation at 95° C for 15 minutes; 35 cycles of: denaturation at 94° C for 1 minute, annealing at 65° C for 1 minute and extension at 72° for 1 minute; final extension at 72° for 10 minutes.

3.2. *g6pd*

The Med mutation (563C>T) in the g6pd gene, the most frequent in Portugal, was the only analysed by amplification of exon 6 followed by digestion with MobII (Thermo Fisher Scientific), as described in Tishkoff *et al*. (2001).

PCR was performed in a final volume of 50 µl with 1.25 units of using Go Taq Flexi® (Promega, Madison, Wisconsin, USA), 1 µM of the primers G6PD_6F and G6PD_6R listed in Table 1, 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 1x Go Taq Flexi Buffer (Promega), GoTaq G2 Flexi (Promega) and 10 ng/µl DNA from the blood samples. The program used in the thermocycler (T1 thermocycler Biometra) was as follows: initial denaturation at 94° C for 1 minute; 30 cycles of: denaturation at 94° C for 1 minute, annealing at 59° C for 1 minute and extension at 72° for 1 minute; final extension at 72° for 10 minutes.

Digestion of 1 μg of the PCR product was performed following the manufacturer's instructions.

3.3. *pklr*

The presence of the most common mutation responsible for PK deficiency in Portugal, 1456C>T at exon 11 (Manco & Abade 2001), was analysed by PCR amplification according to Baronciani and Beutler (1993), followed by Sanger sequencing.

Briefly, the amplification of exon 11 was performed using in a final volume of 50 μl with 1.25 units of Go Taq Flexi® (Promega), 1 μM of the primers pk11R and pk11D listed in Table 1, 200 μM of dNTPs, 1 mM of $MgCl_2$, 1x Go Taq Flexi Buffer (Promega), GoTaq G2 Flexi (Promega) and 10 ng/μl DNA form the blood samples. The program used in the thermocycler (T1 thermocycler Biometra) was as follows: initial denaturation at 94° C for 5 minutes; 30 cycles of: denaturation at 94° C for 45 seconds, annealing at 63° C for 45 seconds and extension at 72° for 1 minute; final extension at 72° for 10 minutes.

QIAxcel Advance (Qiagen) multicapillary electrophoresis instrument was used to verify the size of all PCR and restriction analysis products.

Table 1 - Oligonucleotide primers used in this study.

Primer	Sequence
WT-AS	ATG GTG CAC CTG ACT CCT GA
WT-CP517	CCC CTT CCT ATG ACA TGA ACT
MUT-AS	CAG TAA CGG CAG ACT TCT CCA
MUT-CP267	GGG TTT GAA GTC CAA CTC CTA
pk11R	GAT ATC TCA GTC TTA GTG
pk11D	AGT GAC ACC TGG AAC TGG
G6PD_6F	TGC AGC TGT GAT CCT CAC TC
G6PD_6D	AGG TGG AGG AAC TGA CCT TG

3.4. SANGER SEQUENCING

pklr amplification products were sequenced using primer pk11R at STAB VIDA (Portugal). Sanger sequencing principle lies that at the end of x cycles, depending on the length of the DNA template, and with enough reagents at least one DNA sequence of every length will be produced with a tagged nucleotide, this nucleotide will stop the sequence once dideoxynucleotides do not possess 3'-OH group necessary to continue the strand extension.

4. *IN VITRO* PARASITE CULTURES

4.1 MAINTENANCE OF *P. FALCIPARUM* CULTURES

In this study, *P. falciparum* strains were grown in culture flasks in an incubator at 37° C with a 5% CO_2 atmosphere with an haematocrit of 5% (in a total volume of 3 ml cRPMI) and a parasitaemia below 4%. Both *P. falciparum* strains were maintained in 10,44 g of Roswell Park Memorial Institute (RPMI) 1640 Medium (Biowest, Nuaillé, France) in 1 L of MiliQ water supplemented with 5.94 g of HEPES (VWR, Radnor, PA, USA), 0.05 g of hypoxanthine (Sigma-Aldrich), 5 g of Albumax (Thermo Fisher Scientific) and 5% of sodium bicarbonate. This solution was then sterilized through a 0.22 µm pore filter with a pH of 7.8. The culture medium was exchanged every 24 hours.

4.2. MONITORING *P. FALCIPARUM* CULTURES

In order to monitor the growth of *P. falciparum* culture, daily thin blood smears were performed. They were then fixed with 100% methanol and stained with 20% Giemsa (Sigma-Aldrich). The smears were examined and parasitaemia determined by optical microscopy at 1000x magnification (Olympus BX40).

4.3. THAWING OF *P. FALCIPARUM* STRAINS

The frozen ampoules with two strains of *P. falciparum* (3D7 – chloroquine sensitive and Dd2 – chloroquine resistant) were removed from liquid nitrogen and placed on a water bath at 37° C until it is thawed. The volume was measured and transferred to

a 10 ml centrifuge tube. Solution A NaCl 12% (w/v) was added dropwise and slowly into the flask, while shaking the tube gently (0.2 ml for 1 ml of pellet) and the mixture was left to rest for three minutes. Solution B NaCl 1.6% (w/v) was added in the same way as before (10 ml for 1 ml of pellet) and the mixture was centrifuged for five minutes at 670.8 x g. The supernatant was then rejected and solution C NaCl 0.9% (w/v) and dextrose 0.2% (w/v) were added (10 ml for 1 ml of pellet). The mixture was centrifuged for 5 minutes at 670.8 x g, the supernatant was rejected, and the pellet re-suspended in cRPMI to obtain a haematocrit of 5%. Both strains were maintained in culture at 37° C, 5% CO_2.

4.4. CRYOPRESERVATION OF *PLASMODIUM FALCIPARUM* STRAINS

To preserve *P. falciparum* strains, a culture with 2-3% parasitaemia with mainly ring-stage parasite was centrifuged for 5 minutes at 670.8 x g, the supernatant was discarded, and the volume of sample was measured. The same volume of the cryopreservation solution (28% glycerol, 4.2% sorbitol and 0.6% NaCl) was added. The solution was transferred to a cryopreservation ampoule and stored in liquid nitrogen.

4.5. SYNCHRONIZATION OF CULTURES

To prepare the cultures for the susceptibility assays, both strains of *P. falciparum* were synchronized with sorbitol. With a parasitaemia of 10%, the culture was centrifuged for 5 minutes at 328.7 x g and the supernatant was discarded. Sorbitol 5% (w/v) (Sigma-Aldrich) was added (10 times the volume of the pellet), the solution was shaken vigorously and incubated 10 minutes at 37° C. The solution was centrifuged for 5 minutes at 328.7 x g to remove the supernatant. Subsequently the solution was washed twice with PBS (Sigma-Aldrich) by centrifugation for 5 minutes at 328.7 x g. A smear was made to verify the presence of ring stage parasites and the absence of schizont stage parasites, the haematocrit was set to 5% with RBC and cRPMI.

The procedure was repeated 6 hours later.

5. PARASITE SUSCEPTIBILITY ASSAYS

5.1. IC50 DETERMINATION

In order to determine the value of IC50 (half–maximal inhibitory concentration) SYBR Green assays were performed according to Machado *et al*. (2016). Parasitaemia (%) was determined by optical microscopy of Giemsa stained thin blood smears. To determine the corresponding IC50, 3D7 and Dd2 strains were previously synchronized as described in the previous section. When ring-stage parasites attained more than 85%, both strains were cultivated with an haematocrit of 3% and a parasitaemia of 1% in a 96 well plate for 24, 48 and 72 hours in the presence of different concentrations of 2,3-DPG (0.25, 0.5, 1, 2, 4, 8, 16mM) and a control without the metabolite. After the period of incubation 100 µl of a solution of SYBR Green (0.001% v/v in PBS) (Thermo Fisher Scientific) was added to each well and the plate was then incubated for 1 hour under standard culture conditions (37^{o} C, 5% CO_2). The plate was then centrifuged for 2 minutes at 2750 x g, the supernatant discarded and cells re-suspended in 100 µl of PBS. Fluorescence – Relative fluorescence units, RFU – was measured in a fluorimeter plate reader Triad TM Series Multimode Detector Dynex Tecnologies (Dynex Tecnologies, Chantilly, VA, USA;excitation 485 nm and emission 535 nm). This assay was performed in triplicate.

5.1.1. Resistance Index

The resistance index (RI) of a product or a drug is defined as the ratio of IC50 of the resistant strain to IC50 of the sensitive strain, in this case the ratio of IC50 of Dd2 to IC50 of 3D7. A RI higher than 100, corresponds to an high level of resistance. A RI lower than 10 indicates an intermediate level of resistance and a RI close to 1 suggests an absence of resistance. (Nzila & Mwai, 2009)

5.2. INVASION AND MATURATION ASSAYS

Invasion and maturation assays were performed in order to determine if the increase of 2,3-DPG concentration affected the invasion and/or maturation of the parasite.

The assay was performed in culture flasks of 3 ml with a haematocrit of 5% starting with a synchronized culture with 0.5% of schizont stages. Cultures were maintained during three cycles of growth (until 144h) without addition of RBCs. The 2,3-DPG concentrations tested were chosen according to the previous IC50 determination. Total and stage-specific parasitaemia were determined by daily parasite counting in Giemsa stained thin blood smears.. This assay was performed in triplicate.

To asses if maturation and invasion of the parasite are differentially affected by the addition of 2,3-DPG to the medium, ratios of invasion (Equation 1) and maturation (Equation 2) were calculated.

Equation 1- Invasion ratio.

$$\text{Invasion ratio} = \frac{\text{parasitaemia of rings Dayx+1}}{\text{parasitemia of schizonts Dayx}}$$

Equation 2 – Maturation ratio.

$$\text{Maturation ratio} = \frac{\text{parasitaemia of schizonts Dayx+1}}{\text{parasitaemia of rings Dayx}}$$

6. ATP LEVELS

ATP levels in the cells were estimated in the above assays (sections 5.1. and 5.2.), also in triplicate, using ATP bioluminescent somatic cell assay kit (Sigma-Aldrich), based on a luciferin-luciferase bioluminescence assay. The amount of light emitted was measured using Triad TM Series Multimode Detector Dynex Tecnologies (Dynex Tecnologies, Chantilly, VA, USA).

According to the manufacturer instructions, in a 96 well plate, 100 µl of ATP Assay Mix Working Solution was added to a well [well 1]. In a separate well [well 2], 100 µl of 1xSomatic Cell ATP Releasing Reagent, 50 µl of MiliQ water and 50 µl of the cell culture sample were mixed and 100 µl of this solution was transferred to the well 1 and the amount of light emitted ($L_{(SAM)}$) immediately measured. In a third well [well 3], 100 µl of 1xSomatic Cell ATP Releasing Reagent, 50 µl of the cell culture sample and 50 µl of ATP standard solution (an internal standard, $ATP_{(IS)}$, in moles) were added. 100 µl of this

solution was added to 100 µl of ATP Assay Mix Working Solution in a well 4 and the amount of light emitted ($L_{(SAM+IS)}$) immediately measured.

The amount of ATP in the cell sample ($ATP_{(SAM)}$, in moles) was calculated by the following equation:

Equation 3 – Formula for calculating the amount of ATP in a cell.

$$ATP_{(SAM)} = \frac{ATP(IS) \times L(SAM)}{L(SAM+IS) - L(SAM)}$$

7. STATISTICAL ANALYSIS

The IC50 value was calculated using a nonlinear regression based on a dose response curve. The compound concentrations (x value) were transformed by using X=Log[X] and plotted against the RFU (y values). The data was then analysed in GraphPad Prism version 8 for Windows (GraphpPad Software, San Diego, CA, USA) by nonlinear regression (sigmoidal dose-response/variable slope equation) to yield the IC50 (the drug concentration that produced 50% of the observed decline from the maximum counts in the drug-free control wells).

The non-parametric Mann-Whitney test, was used to compare two unmatched groups and a significance level of 0.05 was considered (P-value < 0.05 is significant).

IV – PRESENTATION AND DISCUSSION OF RESULTS

1. BLOOD GENOTYPING

Previously to parasite susceptible assays it was necessary to exclude a presence of any genetic disorders related to haemoglobin (search for the sickle-cell trait, HbS) or erythrocyte enzymes (G6PD and PK) that could influence the outcome of parasite susceptibility assays.

Regarding the gene HbB, the wild type primer set (pair WT-AS / WT-CP517) amplify a fragment of 517 bp corresponding to the HbA allele, the mutant primer set (MUT-AS / MUT-CP267) amplify a fragment of 267 bp corresponding to HbS allele. A heterozygote (HbAS) is identified by the presence of both PCR products. In all samples, the target region amplified by non-allele-specific outer primers (WT-CP517 / MUT-CP267) should be amplified as an internal control, originating a fragment of 800 bp.

Results obtained with the five blood donors are depicted in Figure 6 (lanes 2 to 6). In all, two fragments are visible - the control fragment of 800 bp and the fragment of 517 bp corresponding to the wild homozygote HbAA. No HbAS or HbSS were found, otherwise would have to be discarded.

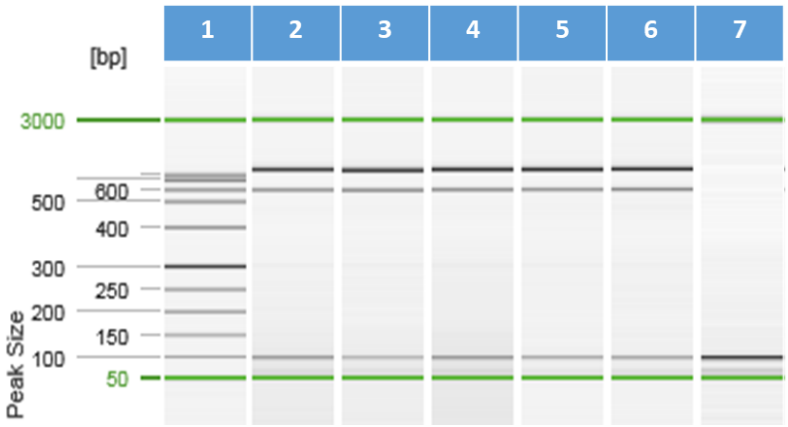

Figure 6 - Amplification of HbA fragments from blood donors by multiplex PCR. Lane 1: QX DNA Size Marker 50-800 bp. Lane 2: Donor 1. Lane 3: Donor 2. Lane 4: Donor 3. Lane 5: Donor 4. Lane 6: Donor 5. Lane 7: Negative control (without DNA).

In the case of *g6pd* gene, the presence of the most common mutation in Portugal, the mutation Med (usually ranges in frequency from 2 to 20% in Southern Europe and is associated to a 3% of the normal enzymatic activity) was analysed by PCR-RFLP.

The amplification of exon 6 originates a fragment of 388 bp as shown in lanes 2-6 of Figure 7. After restriction with the MboII enzyme, two fragments of 352 and 36 bp are expected if mutation is absent and three fragments of 253, 99 and 36 bp if mutation is present.

In Figure 8 (where amplified fragment of exon 6 is compared with the respective digested product), a fragment of 352 bp is visible for all blood donors; we assume that the band of 36 bp is not visible due to the small fragment size and mutation does not occur in none of them.

Figure 7 - Amplification of *g6pd* gene from blood donors by PCR. Lane 1: QX DNA Size Marker 50-800 bp. Lane 2: Donor 1. Lane 3: Donor 2. Lane 4: Donor 3. Lane 5: Donor 4. Lane 6: Donor 5. Lane 7: Negative control (without DNA).

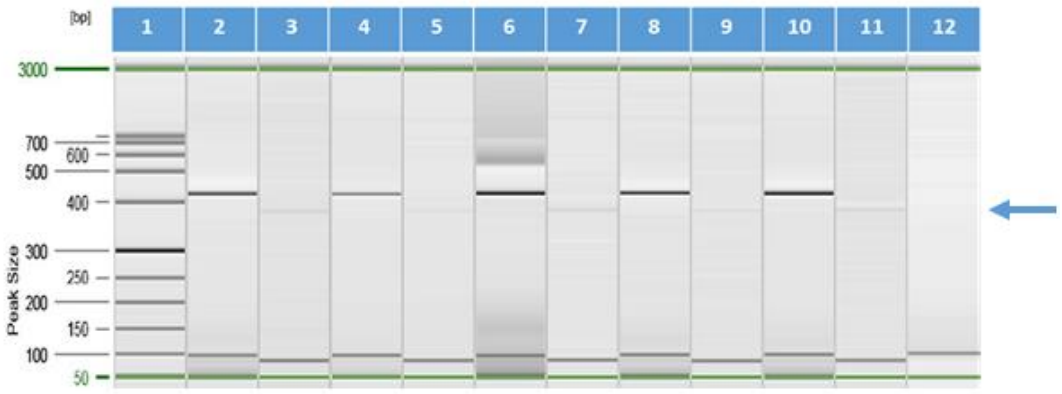

Figure 8 - Restriction analysis of *g6pd* gene from blood donors. Lane 1: QX DNA Size Marker 50-800 bp. Lane 2: Donor 1. Lane 3: Donor 1 digested with MboII. Lane 4: Donor 2. Lane 5: Donor 2 digested with MboII. Lane 6: Donor 3. Lane 7: Donor 3 digested with MboII. Lane 8: Donor 4. Lane 9: Donor 4 digested with MboII. Lane 10: Donor 5. Lane 11: Donor 5 digested with MboII. Lane 12: Negative control (without DNA). Arrow corresponds to the size of the digested fragment with 352 bp.

Regarding the exon 11 of *pklr* gene, according to Baronciani and Beutler (1993), a fragment of 413 bp is expected. However, at the beginning, multiple fragments were obtained (Figure 9). In order to optimize and increase the specificity of the reaction, several concentrations of $MgCl_2$ were tested (1, 1.5, 2 and 3 mM). Based on the results shown in Figure 10, the concentration of 1 mM was chosen to test the other blood donors (Figure 11).

The presence of the 1456C>T mutation is associated to the disappearance of the BsmAI cleavage site in the amplified products. As a good positive control for a mutated individual was not available, the amplified fragments were sequenced and the presence of the restriction enzyme site 5' GTCTC 3' was confirmed in all blood donors.

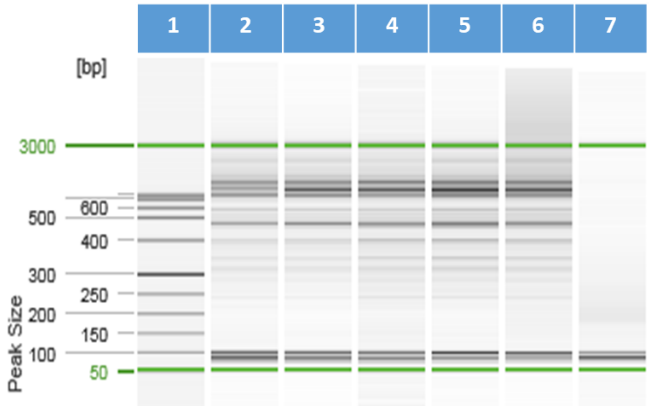

Figure 9 - Amplification of *pklr* gene from blood donors by PCR. Lane 1: QX DNA Size Marker 50-800 bp. Lane 2: Donor 1. Lane 3: Donor 2. Lane 4: Donor 3. Lane 5: Donor 4. Lane 6: Donor 5. Lane 7: Negative control (without DNA).

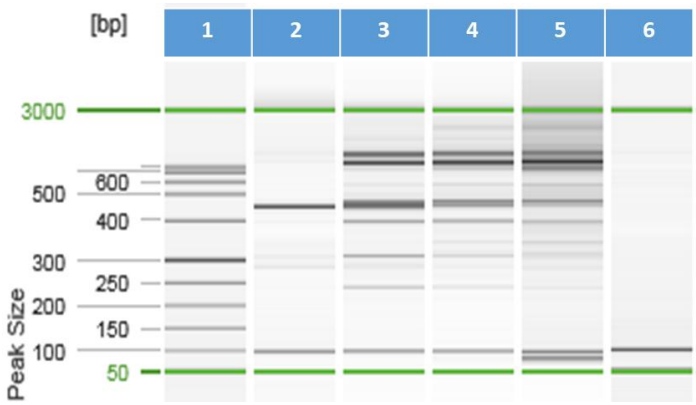

Figure 10 - Amplification of *pklr* gene from blood donor 1 by PCR using different concentrations of $MgCl_2$. Lane 1: QX DNA Size Marker 50-800 bp. Lane 2: 1 mM of MgCl2. Lane 3: 1.5 mM of MgCl2. Lane 4: 2 mM of MgCl2. Lane 5: 3 mM of MgCl2. Lane 6: Negative control.

Figure 11 - Amplification of *pklr* gene from blood donors by optimized PCR protocol. Lane 1: QX DNA Size Marker 50-800 bp. Lane 2: Donor 1. Lane 3: Donor 2. Lane 4: Donor 3. Lane 5: Donor 4. Lane 6: Donor 5. Lane 7: Negative control (without DNA).

Have confirmed the absence of genetic disorders related to the β-globin, *g6pd* and *pklr* genes of the blood donors, we consider the RBCs suitable to proceed for the *in vitro* cultures and parasite susceptibility assays.

2. PARASITE SUSCEPTIBILITY ASSAYS

2.1. IC50 DETERMINATION

To determine the IC50 of 2,3-DPG, chloroquine sensitive strain (3D7) and chloroquine resistant strain (Dd2) were analysed during 24, 48 and 72 hours in the presence of different concentrations of the compound.

In the first assay, the *P. falciparum* cultures were not synchronised and both ring and schizont stages were present. Figure 12 shows the dose-response curves respecting different concentrations of 2,3-DPG in an unsynchronised culture, where very high standard deviation values can be observed. Further, in some cases (two assay of 3D7 at 24 hours, Dd2 at 24 and 48 hours), the IC50 value cannot be determined once the dose response curve is not a sigmoid curve but a straight line, making IC50 calculation not possible (Table 2).

To confirm the result and to exclude operators' error, the same assay with 3D7 was performed simultaneously with 2,3-DPG and chloroquine for which the IC50 value is well documented. The IC50 values obtained for chloroquine (Figure 13, Table 3) are consistent with the results of Nogueira *et al*. (2008) at 48 hours (IC50 value of 16 nM for 3D7 and 116 nM for Dd2), similar to the IC50 values obtained in the present work (17 nM for 3D7 and 244 nM for Dd2). Despite the difference for Dd2, IC50 values are in the same order of magnitude. The values obtained for chloroquine validate the results attained for 2,3-DPG in an unsynchronized culture. It can be observed that the IC50 values obtained decreased through time due to the cumulative effect of chloroquine in the culture medium, by increasing the accumulation of heme monomers that permeabilize the membrane leading to the dead of the parasite. Therefore, a lower concentration of chloroquine is required to produce a 50% inhibition of *P. falciparum in vitro* at 72 hours, when compared to an incubation period of 24 hours.

Due to the lack of consistence in the results, it was hypothesized that the effect could be stage-specific and a certain parasite stage could be more susceptible than others to the increased concentration of 2,3-DPG in the culture medium. The assay for the determination of IC50 was then performed with a synchronized culture (rich in ring stage parasite at hour 0) (Figure 14).

It is observed that IC50 values at 24 and 72 hours for both strains (Table 4) are within the normal values of 2,3-DPG in RBCs. This was unexpected since the normal levels of 2,3-DPG in RBCs are not harmful for the parasite; if this was to be true everyone would have a mechanism to resist to *Plasmodium* spp. infection. However, these results obtained in an *in vitro* assay cannot be directly extrapolated for an *in vivo* model. *In vivo*, 2,3-DPG regulates the affinity between oxygen and haemoglobin, and the normal saturation can vary but it is normally above 94% while *in vitro*, parasites are grown under low oxygen conditions, around 1 to 5%. (Langley & Cunningham, 2017; LeRoux *et al*., 2019) 2,3-DPG could be more toxic *in vitro* than *in vivo*, as it would be more available due to the low oxygen tension in culture.

It was previously mentioned that the chloroquine IC50 values decrease through time, the same thus not occur with 2,3-DPG. At 48 hours the concentration of 2,3-DPG required to inhibit the growth of 50% of *P. falciparum* culture is higher than at 24 hours, the effect of 2,3-DPG in culture medium seems to be cumulative from 24 to 72 hours, when a majority of schizonts stage parasites are present in culture. From 0 to 24 hours of incubation the ring-stage parasite undergo cell division to form trophozoites. During this period is hypothesized that 2,3-DPG is metabolised at an higher rate due to an increased metabolic rate in this phase. An hypothesis for the fact that IC50 values are higher at 48 hours may be due to the decreased metabolic rate due to maturation and consequently the decreased metabolization of 2,3-DPG. Nevertheless, this seems to indicate that the effect of 2,3-DPG in culture medium varies with the parasite stage present in culture.

The resistance index, RI, was calculated for 2,3-DPG for the different times of incubation (Table 3) and was compared with the RI for chloroquine (Table 4). The different values of RI for 2,3-DPG are lower than 2 and in the case of 24 hours incubation the RI value is 0.40 meaning that the IC50 value of Dd2 is lower than for 3D7. While the RI of chloroquine varies between 7.77 and 30.33, which is consistent with a considerable resistance of the Dd2 strain, for 2,3-DPG, both strains seem to respond in the same way and no resistance is visible.

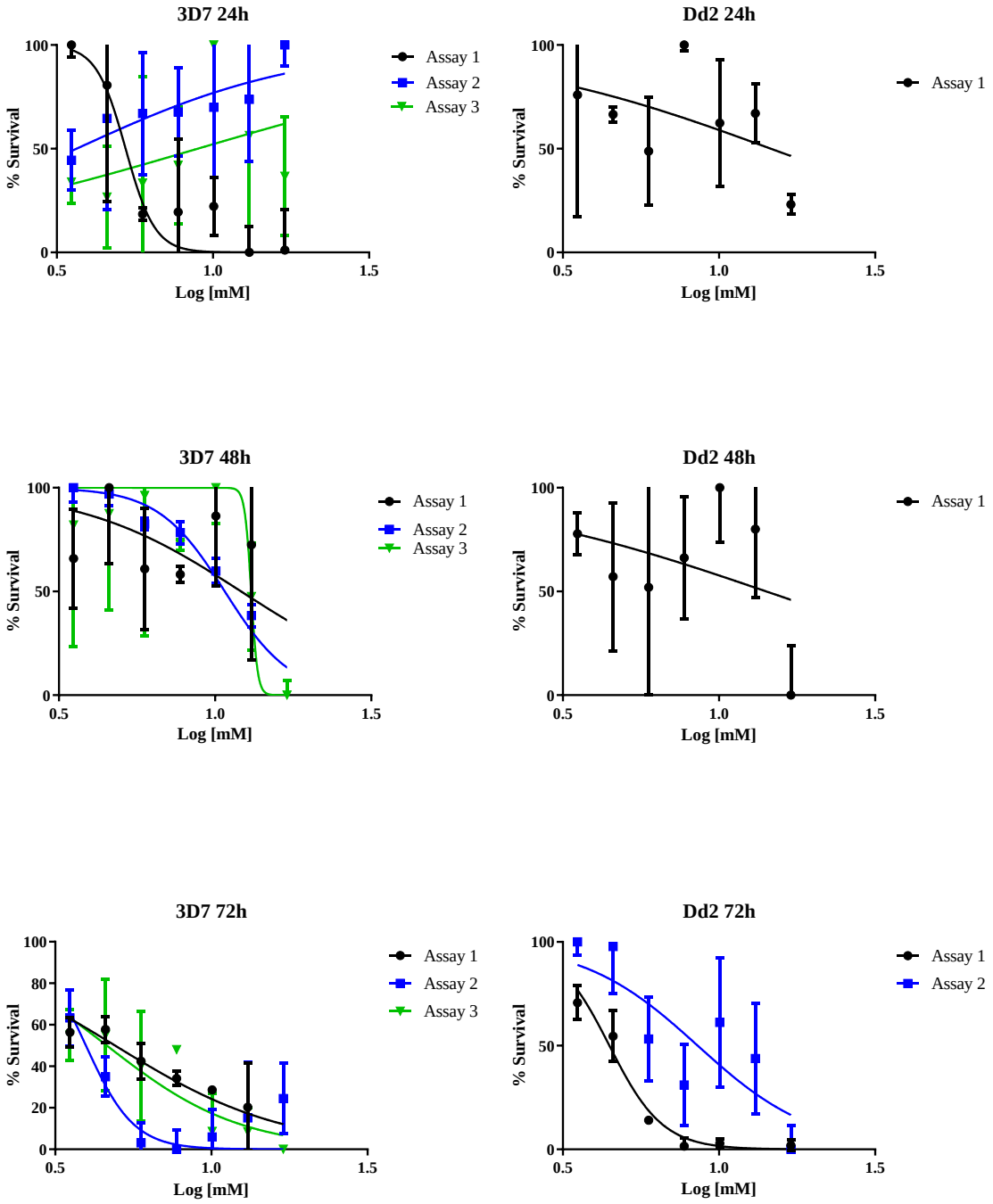

Figure 12 – Dose response curves for unsynchronized cultures of 3D7 and Dd2 strains after 24, 48 and 72 hours of incubation with 2,3-DPG. The different colors correspond to different assays.

Table 2 - IC50 values and corresponding standard deviation obtained for unsynchronized cultures of 3D7 and Dd2 strains after 24, 48 and 72 hours of incubation with 2,3-DPG. IC50 value calculation for Dd2 at 24 and 48 hours was not possible.

	3D7	Dd2
24 hours	5.25 mM	———
48 hours	11.97 ± 0.95 mM	———
72 hours	4.50 ± 0.40 mM	6.50 ± 2.04 mM

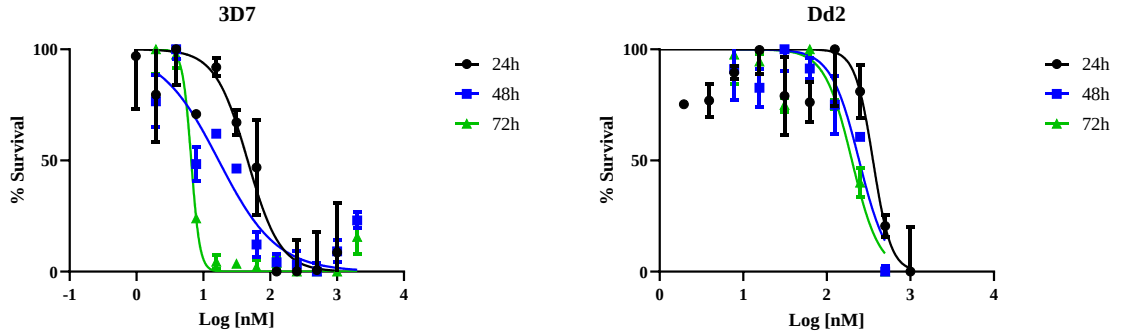

Figure 13 – Dose response curves for unsynchronized cultures of 3D7 and Dd2 strains after 24, 48 and 72 hours of incubation with chloroquine. The different colors correspond to different incubation times.

Table 3 - IC50 values and corresponding standard deviation obtained for unsynchronized cultures of 3D7 and Dd2 strains after 24, 48 and 72 hours of incubation with chloroquine. RI (Resistance Index), ratio between IC50 of Dd2 and IC50 of 3D7.

	3D7	Dd2	RI
24 hours	46.03 nM	357.7 nM	7.77
48 hours	16.97 nM	244.0 nM	14.38
72 hours	6.59 nM	199.9 nM	30.33

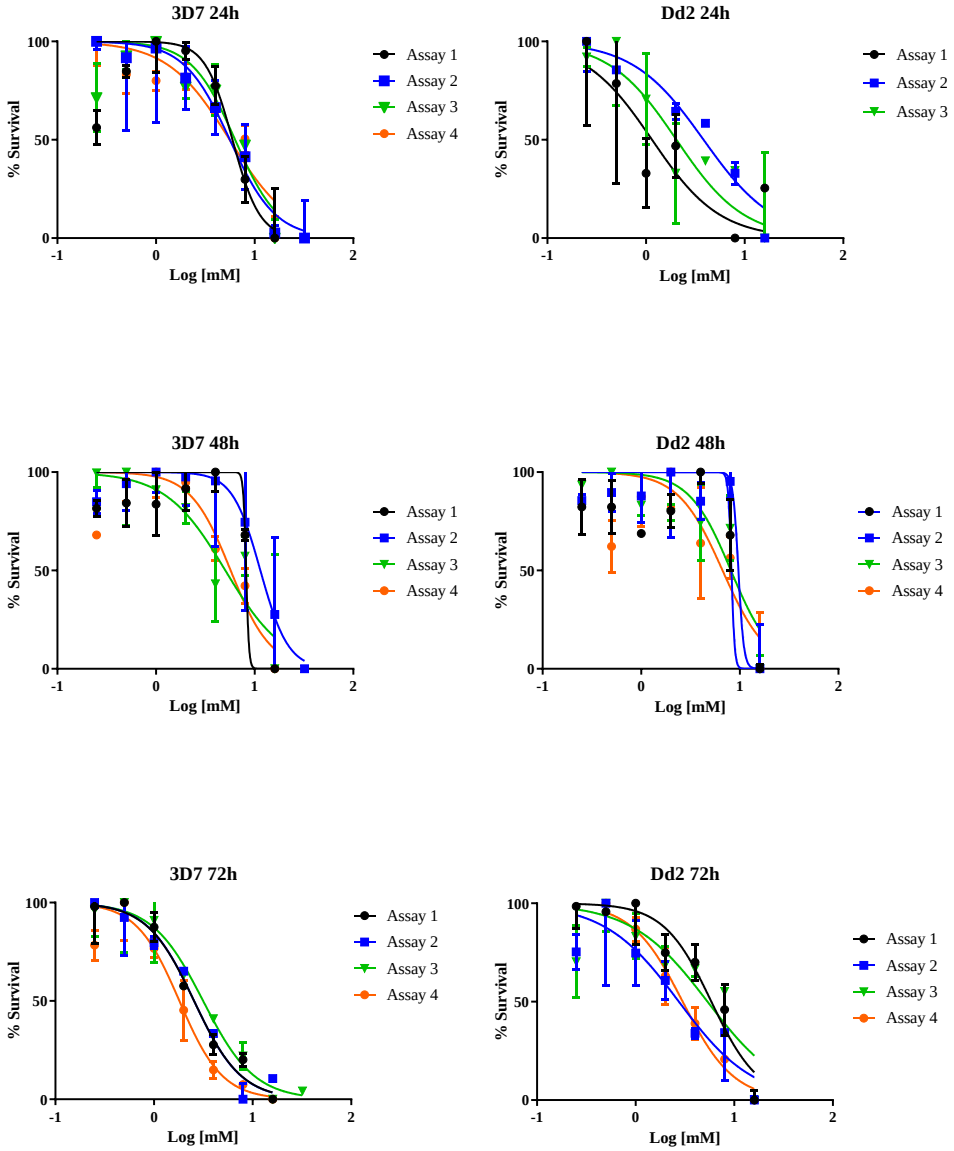

Figure 14 – Dose response curves for synchronized cultures of 3D7 and Dd2 strains after 24, 48 and 72 hours of incubation with 2,3-DPG. The different colors correspond to different assays.

Table 4 - IC50 values and corresponding standard deviation obtained for synchronized cultures of 3D7 and Dd2 strains after 24, 48 and 72 hours of incubation with 2,3-DPG. RI (Resistance Index), ratio between IC50 of Dd2 and IC50 of 3D7.

	3D7	Dd2	RI
24 hours	5.87 ± 0.31 mM	2.34 ± 1.12 mM	0.40
48 hours	7.60 ± 2.51 mM	8.18 ± 1.06 mM	1.08
72 hours	2.52 ± 0.48 mM	4.24 ± 1.38	1.68

2.2 INVASION AND MATURATION ASSAYS

Based on the IC50 results which ranged from 2.34 ± 1.12 mM to 8.18 ± 1.06 mM, three concentrations of 2,3-DPG – 3, 5 and 8 mM – were chosen to examined if *P. falciparum* parasites invaded and matured as efficiently in RBCs with 2,3-DPG in the medium as in control (without 2,3-DPG).

Daily total and stage-specific parasitaemia were determined during three cycles of growth (144 hours). During the parasite growth cycles, the synchronization of culture and the alternation of parasite stage, between ring-stage to trophozoite/ schizont-stage, can be observed (Figures 15 – predominance of ring stages at 24, 72 and 120 hours; predominance of trophozoite/schizonts stages at 48, 96 and 144 hours). Figure 15 is illustrative of the effect of 2,3-DPG in culture medium (5 mM) throughout time on parasitic growth, especially when compared with the control. At 120 and 144 hours, the difference in parasitaemia can be seen in images as having an higher parasitaemia in the control.

When the total parasitaemia is analysed (Figure 16 and 17), a similar growth rate is observed both in the control and with the 2,3-DPG doses during the first cycle (until 48h). From 72h onwards, although not significantly, the difference increases with higher parasitaemia in the control ($p=0.1$ for 3D7 and $p=0.1$ for Dd2); parasitaemia peaks at 96h for both strains and at 168h, RBC lysis occurs and the culture die.

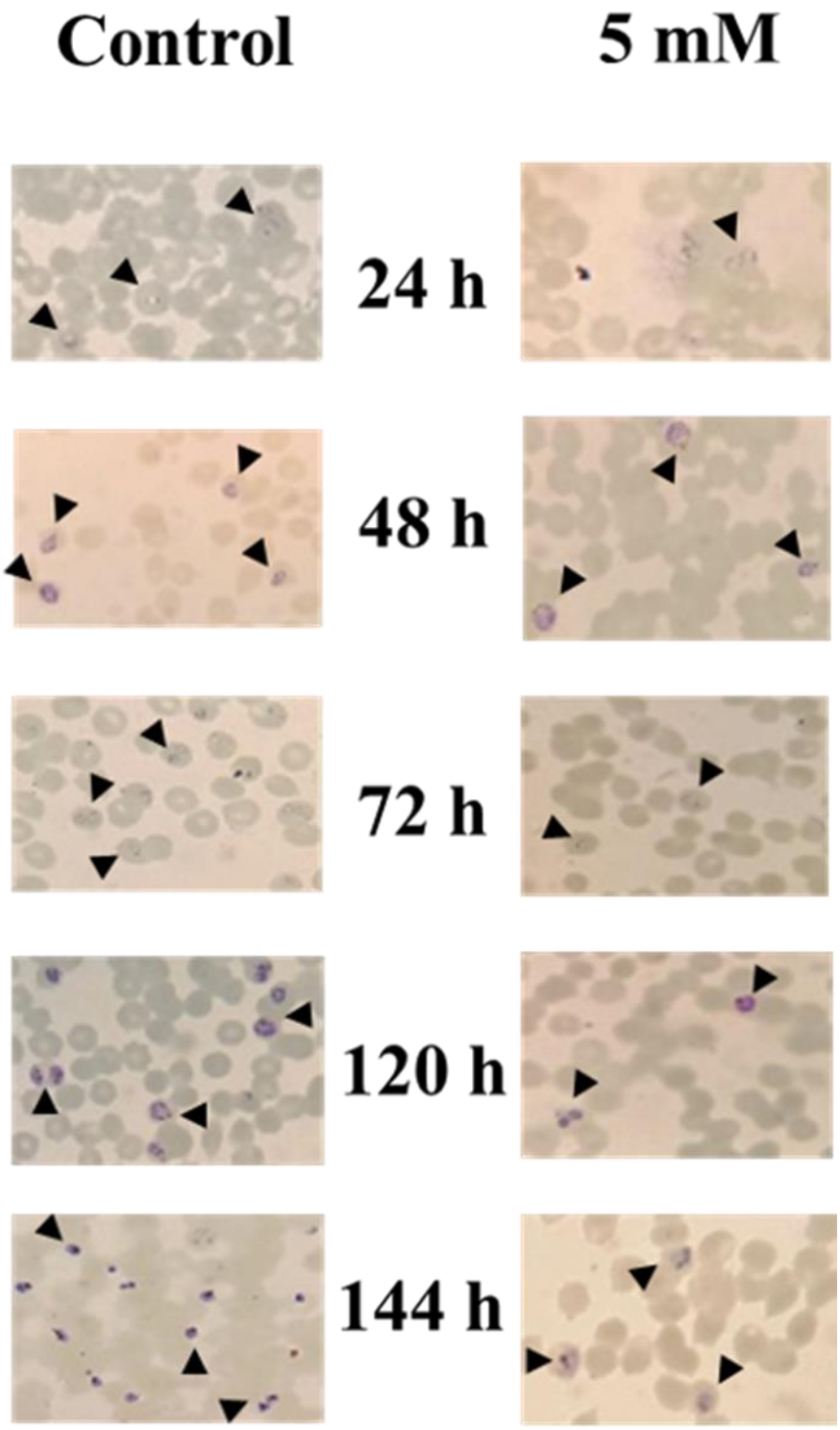

Figure 15 –Giemsa-stained thin blood smears of 3D7 strain growing without 2,3-DPG (control) and incubated with a 5 mM concentrations of 2,3-DPG over time (three cycles, 24-144 hours). Arrows indicate the parasite. Predominance of ring stages at 24h, 72h and 120h; predominance of trophozoite/schizonts stages at 48h, 96h and 144h.

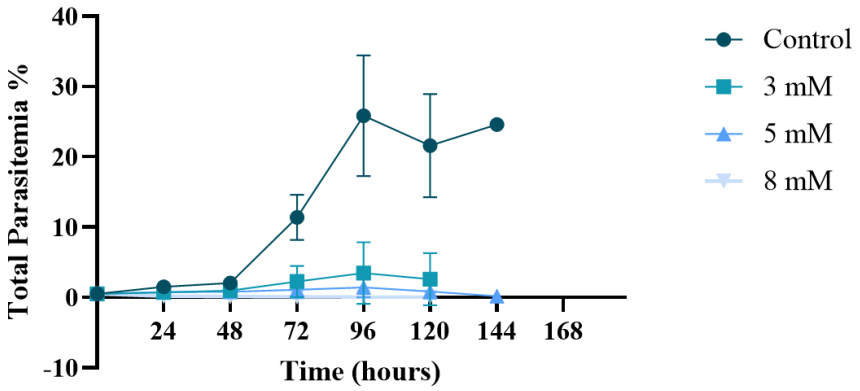

Figure 16 - Total parasitaemia of *Plasmodium falciparum* 3D7 during three cycles of growth.

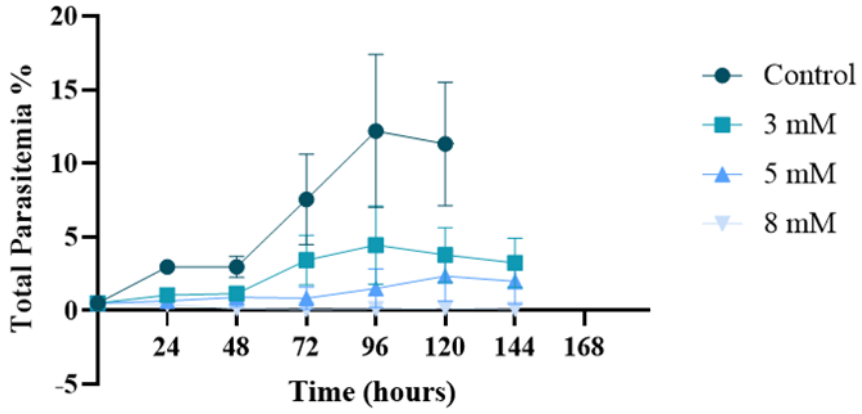

Figure 17 - Total parasitaemia of *Plasmodium falciparum* Dd2 during three cycles of growth.

Although a trend for higher maturation and invasion ratios in control than in the 2,3-DPG concentrations is observed, differences are not significant. The difference is more remarkable regarding invasion (Figure 18 and 20) than maturation ratios (Figure 19 and 21). Strains behave slightly differently since Dd2 appears to be more affected early in the first cycle than 3D7; on the second cycle, with Dd2 the same effect is visible only with 5 mM and 8 mM of 2,3-DPG.

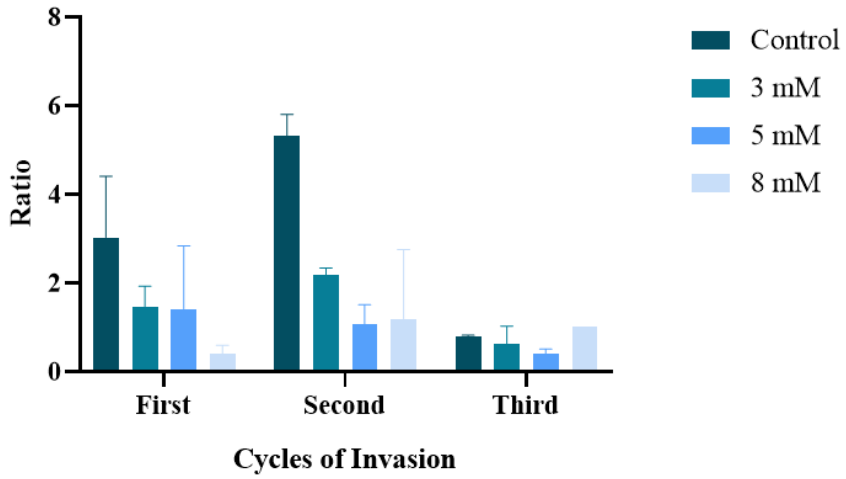

Figure 18 - Invasion ratios of 3D7 during three cycles of invasion.

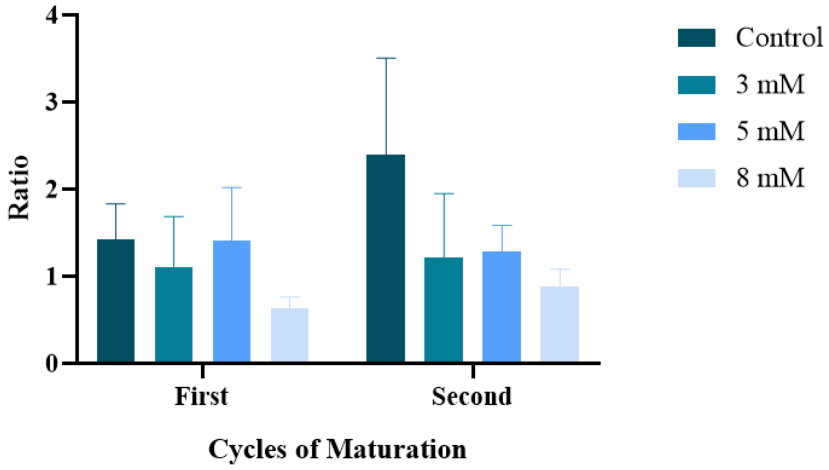

Figure 19 - Maturation ratios of 3D7 during two cycles of maturation.

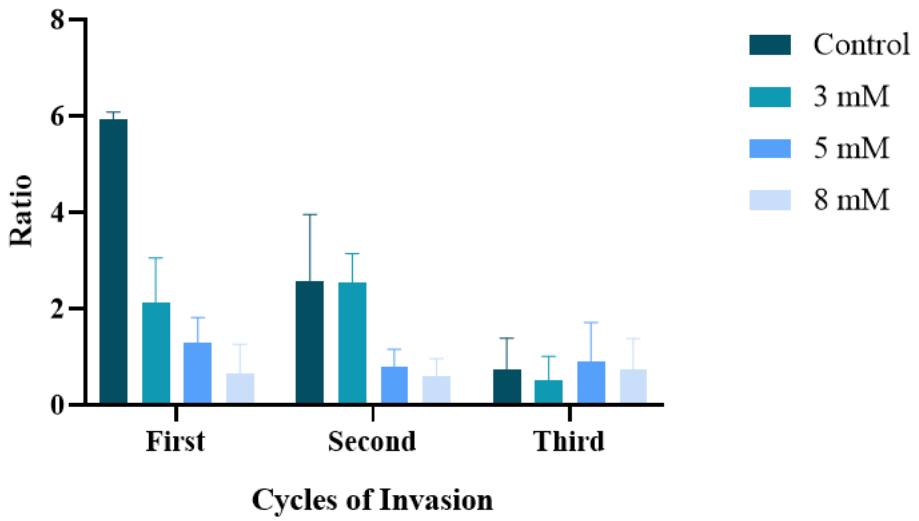

Figure 20 - Invasion ratios of Dd2 during three cycles of invasion.

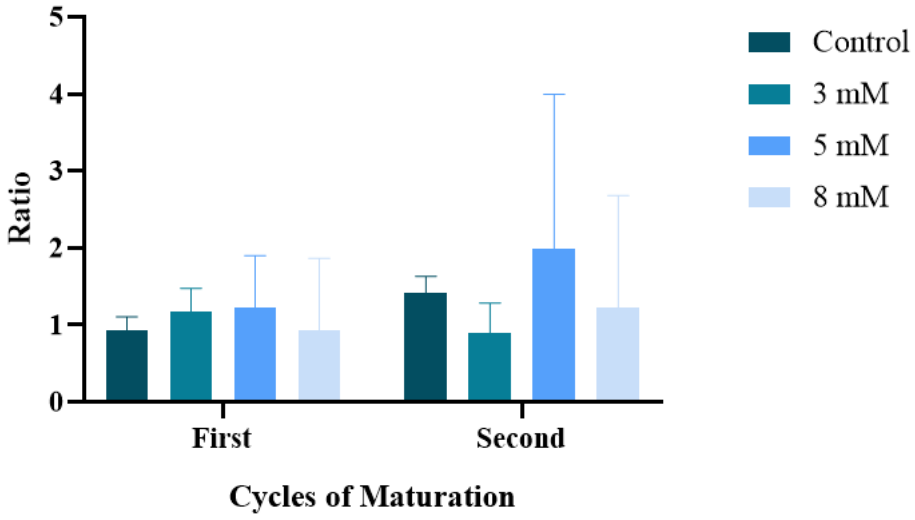

Figure 21 - Maturation ratios of Dd2 during two cycles of maturation.

Results suggest a reduction in the RBCs invasion by the parasite when the metabolite 2,3-DPG is added to the culture medium. However, as the parasitaemia was determined every 24 hours, we cannot be sure if it is the merozoite that is affected when invading the RBC. As maturation ratios do not appear to be affected, ie if the parasite invades, it can normally mature to late trophozoite/schizont, it may also happen that new merozoites are not able to egress the RBC, which would imply less invasion and lower ring-stage counts in the next cycle of growth. This result may be clarified if parasite counting could be done in shorter intervals such as 6 or 12 hours and a more accurate determination of parasitaemia by flow cytometry is carried out; it would also help to verify if the differences seen between strains, even not significant, are real or an artefact.

Either a difficulty in invasion or in egress occurs, it appears that the effect is felt more in the involvement of the RBC cell membrane than in the parasite itself. It could be interesting to perform the same assay with well-known antimalarial drug or another compound as a reference. CQ may have an effect on parasite maturation rather on invasion but a compound like calpain-1 that dismantles the cytoskeleton and leads to RBC membrane collapse could be used. (Millholland *et al*. 2011)

Ayi *et al*. (2008) studied the growth of *P. falciparum* in *in vitro* cultures using PK deficient RBC and observed lower infection and replication. The authors suggested that the reduction on the invasion ratio may not be a consequence of the parasite fitness, but instead it could be due to alterations in RBC membranes.

As stated before, in the present study, we try to understand if the increase of the intermediary metabolite 2,3-DPG, a consequence of PK deficiency, could be the mechanism underlying that effect. An increase in 2,3-DPG concentration affects the stability of RBCs membrane as a concentration of 2,3-DPG of 4 mM in the medium results in a 60% dissociation of spectrin tetramer to dimer. (Sheetz & Casaly, 1981) The dissociation of spectrin will affect two protein complexes – ankyrin and 4.1R – that are responsible for the linkage between the lipid bilayer and the spectrin-based skeleton network. This results in a decreased deformability of erythrocytes that can lead to greater difficulty for the parasite to cross the RBCs membrane. In order to characterize changes on RBCs morphology and cellular membrane, Atomic Force Microscopy (AFM) should

be considered to compared infected and uninfected RBCs in the absence and presence of different concentrations of 2,3-DPG.

3. ATP LEVELS

ATP levels in the cells as determined with the ATP bioluminescent somatic cell assay kit, do not vary in a consistent way, whether in the case of IC50 determination (Table 5) or in invasion and maturation assays (Table 6). Figure 22 illustrates the evolution of ATP levels with increased concentration of 2,3-DPG in the culture medium at 72h in the IC50 assay.

A decrease in the concentration of ATP as the 2,3-DPG concentration increases would be expected as it may be seen in Table 5, no-infected RBC at 24h and 48h but the result is not regular, so it is not conclusive.

It is documented that an augmented concentration of 2,3-DPG results in a decreased production of ATP as a higher concentration of this metabolite increases the activity of the Rapoport-Luebering shunt by bypassing the synthesis of ATP the rate-limiting step of glycolysis catalysed by PGK where phosphoenolpyruvate is converted to pyruvate with the generation of one molecule of ATP. (van Wijk & van Solinge, 2005) However, this would be inside RBC and in these assays the 2,3-DPG is added to the medium and these artificial conditions even if affect the RBC membrane as hypothesised above, do not mimic the intracellular environment adequately if 2,3-DPG does not properly enter the erythrocyte.

To overcome these limitations, it would be interesting to mimic the PK deficiency via inhibition of glycolysis pathway in a closer step. Enolase is a good candidate as the inhibition of this enzyme would lead to the accumulation of 2-phosphoglycerate and consequently a reversal on the glycolytic pathway, ultimately resulting in an higher synthesis of 2,3-DPG. These results would be useful to compare with the results obtained in this master thesis, in order to understand if there is a difference in the efficacy of the different approaches. Decreased concentration of ATP leads to cross-linking of membrane proteins, which also may affect invasion and egress of the parasite. (Durand & Coetzer, 2008)

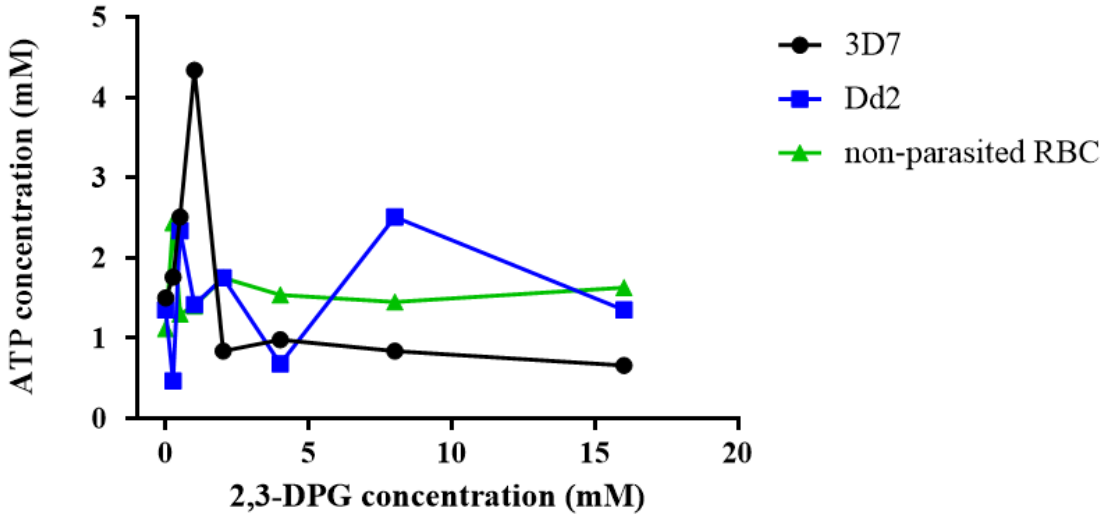

Figure 22 - Influence of 2,3-DPG on ATP levels on 3D7, Dd2 and non-infected RBCs at 72 hours of incubation, during the IC50 assay determination.

Table 5 - Influence of 2,3-DPG on ATP levels on 3D7, Dd2 and non-infected RBCs during the IC50 assay. ATP concentrations are in mM.

2,3-DPG (mM)	3D7			Dd2			RBC		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
16	1.43	1.82	0.66	4.83	0.45	1.35	0.66	0.63	1.63
8	1.28	3.27	0.84	1.65	1.27	2.51	0.78	0.63	1.45
4	1.38	2.50	0.98	0.28	4.22	0.68	0.84	0.641	1.54
2	1.77	2.56	0.84	2.64	2.46	1.75	0.71	0.52	1.75
1	1.52	2.38	4.34	3.02	1.74	1.42	1.86	1.69	1.40
0.5	1.52	1.93	2.51	0.66	1.80	2.34	2.66	2.85	1.30
0.25	2.16	2.79	1.76	4.03	2.34	0.47	2.72	2.17	2.44
0	0.42	0.47	1.50	4.83	0.45	1.35	3.44	2.49	1.12

Table 6 - Influence of 2,3-DPG on ATP levels on 3D7 and Dd2 during the invasion-maturation assays. ATP concentrations are in mM.

Incubation time	3D7				Dd2			
	Control	3 mM	5 mM	8 mM	Control	3 mM	5 mM	8 mM
24h	3.85	2.75	4.02	3.77	5.97	2.33	2.73	2.86
48h	6.28	3.27	1.32	2.96	4.75	6.26	4.55	1.43
72h	1.87	3.75	1.11	3.99	2.45	2.06	1.92	2.23
96h	0.75	1.37	1.26	2.63	0.57	2.59	5.10	0.79
120h	0.19	0.81	0.34	1.02	1.48	1.67	0.29	0.93

V – FINAL CONSIDERATIONS

This study is integrated in a major project that aims to assess the effect of the glycolytic intermediary metabolite 2,3-DPG on the development of malaria parasite, *P. falciparum* assuming as an hypothesis that it may consist in the main mechanisms of PK deficiency protective effect. If these mechanisms of protection could be understood, they could be adapted and used as new tools and antimalarial agents.

Overall, results obtained both at parasite susceptibility assays and invasion and maturation assays are encouraging as seem to point to an effect on the parasite (IC50) and/or RBC membrane affecting the cell invasion (or egress). However, differences were not statistically significant, and a different approach should be tested, namely, to use a more accurate method for parasitaemia determination (eg flow cytometry) as well as a reduction on the time frame of measurements.

If 2,3-DPG could be adapted to an antimalarial agent in the future, it would have the advantage of being less toxic to human cells than most antimalarials as IC50 values obtained are within the normal concentration of 2,3-DPG on RBCs. Nevertheless, an increase of 2,3-DPG concentration in RBC may also imply a certain toxicity for the human cell, as it destabilizes the cell membrane and interferes with ATP production. Therefore, it would be important to test the effect of 2,3-DPG on human cells by performing hepatotoxicity and haemolysis assays.

The analysis of RBC membrane by AFM and analysis of metabolic extracts from infected and non-infected RBC under the effect of different concentrations of 2,3-DPG will be performed in the scope of the project where this study is included and will help to clarify some of the results obtained. Further, the fact that concentration of 2,3-DPG is artificially increased by addition in the culture medium and not inside the cells is a limitation. In order to mimic the effect of this increase inside the cell as result of a deficient PK, this enzyme or a closer one in the glycolytic pathway should be inhibited.

Still, there are other important and long-term questions to be weighted. How does 2,3-DPG perform *in vivo*? Is *P. falciparum* able to gain resistance to 2,3-DPG? And, not less important, in the case of 2,3-DPG be considered a good candidate to an antimalarial, would it be affordable? Most countries where malaria is endemic are low income countries so would be possible to use 2,3-DPG as an antimalarial agent in countries with low financial possibilities?

VI – REFERENCES

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VII – ANNEX

CONSENTIMENTO INFORMADO, LIVRE E ESCLARECIDO
para participação em projeto de investigação

Por favor leia com atenção a seguinte informação. Se achar que algo está incorreto ou pouco claro, não hesite em solicitar mais informações junto do coordenador do projeto. Se concordar com a proposta que lhe foi feita, queira assinar este documento.

Título do Projeto | Quando a célula hospedeira já não é tão acolhedora... Uma quebra de energia ou um aumento da toxicidade?
Instituição Promotora | Instituto de Higiene e Medicina Tropical (IHMT)
Contacto da Coordenadora do Projeto | Ana Paula Arez (aparez@ihmt.unl.pt, +351213652600 ext. 361)

Este projeto tem como objetivo investigar o controlo da via glicolítica em eritrócitos parasitados e o papel do metabolito intermediário 2,3-DPG, visando uma utilização futura como agente antimalárico, e inclui uma componente de experimentação laboratorial utilizando culturas contínuas *in vitro* de Plasmodium falciparum - parasita causador de malária. As culturas são efetuadas em hemácias humanas, obtidas a partir de indivíduos voluntários saudáveis (geralmente membros da própria equipa ou da mesma unidade). O sangue total (20ml) é colhido por punção venosa em EDTA ou citrato e imediatamente lavado com meio de cultura para remover o plasma e leucócitos. Uma pequena alíquota é utilizada para proceder ao despiste de fatores hematológicos que possam inviabilizar a sua utilização como células controlo, tais como polimorfismos nos genes HbAS, g6pd, pklr e tpi. Apenas as hemácias são mantidas em meio de cultura a 4º ou na própria cultura. Idealmente, as células serão usadas nas 24 horas seguintes à colheita e descartadas no máximo, 2 semanas depois.

Não haverá tratamento de dados individuais nem qualquer questionário associado à doação de sangue. A sua participação é voluntária e não envolve qualquer tipo de compensação monetária. Não existem riscos ou desconfortos previsíveis por participar neste estudo. Apenas a coordenadora do estudo terá acesso à correspondência entre o código da colheita e a base de dados com o seu nome e contacto.

Ainda que possa ser-lhe solicitada a doação de sangue mais do que uma vez, pode desistir da participação no estudo sem qualquer consequência, para o que é suficiente contactar a coordenadora do estudo através do email.

Os resultados do estudo serão tornados públicos em reuniões e publicações científicas. Se desejar que os resultados lhe sejam enviados queira por favor contactar a coordenadora do estudo por email.

Até cinco anos após a publicação dos resultados deste estudo, os códigos que ligam a sua identidade e contactos à informação fornecida serão destruídos assim como este formulário que agora assina.

Este estudo foi aprovado pelo Conselho de Ética do IHMT.

AGRADECEMOS A SUA COLABORAÇÃO

Assinatura do(a)
Investigador(a)_____

Declaro ter lido e compreendido este documento, bem como as informações verbais que me foram fornecidas pela pessoa que acima assina. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, aceito participar neste estudo e permito a utilização do material biológico por mim doado voluntariamente, confiando nas garantias de confidencialidade e anonimato que me são dadas pela equipa de investigação.

Nome:_____

Assinatura:_____

Data: ..... /..... /.....

ESTE DOCUMENTO É COMPOSTO DE 1 FOLHA E FEITO EM DUPLICADO: UMA VIA PARA O INVESTIGADOR, OUTRA PARA A PESSOA QUE CONSENTE